Recent Advances in Scientific Research and Technology

Editors

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Dr. Balakrishnan Ramanathan

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Dr. Ramanathan is the Founder and Managing Director of ACE International Pte Ltd, Singapore. He has more than 20 years of experiences in Research & Development, Learning & Development and Business & Development sectors. His research working experience includes biological systems based research and development in basic and clinical medicine areas in a number of leading institutions including: Bhrarathidasan University (India), Indian Institute of Science (India), Chang-Gung University (Taiwan), Academia Sinica (Taiwan), National University of Singapore (Singapore), National Neuroscience Institute (SingHealth), Taipei Veterans General Hospital, National Yang-Ming University (Taiwan). His research expertise are related to immunocontraceptive vaccine development, antibody production, proteomics, glyco-immunochemistry, genotoxicity, DNA damage and repair, anticancer drug development, free radical biology medicine and cell signaling pathways, stem cell research and applications. During his career he has published many peer reviewed articles, provided lead talks in seminars and conferences, acted as mentors for research projects and received several awards and recognitions to his academic and research credits. Currently, he engages the business management consultancy services and career support services, technology transfer and establishing the business network, interdisciplinary translational research and development programs. He is also passionate to conduct motivational talks, capability building workshops, trainings, coaching and research project mentorships. He is active in LinkedIn, Facebook, YouTube and few other social media platforms.

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Article 1

Study on Essential Oils as Repellents for Haemadipsa

Shiny K J and Jilu M G

Abstract

Leeches are sangivorous annelids seen in humid climate. They feed on blood of mammals including man. People working in leech infested agriculture and plantation areas as well as tourists suffer from leech bites. The skin may be injured and ulceration may follow. Mouth parts that remain can cause infection. The locals apply salt to remove leech. It gets washed away easily and is effective only after the leech attack. Essential oil repellents are a safe alternative. Leech repellent properties of some essential oil were evaluated in this study.

The study was conducted in Ponkunnam area of Kottayam district, Kerala. The essential oils selected for this study were -basil oil, ginger oil, vetiver oil, turmeric oil, lemongrass oil, cinnamon oil, clove bud oil and coconut oil. The essential oils were mixed with coconut oil and applied on the legs of volunteers against leeches. The time taken to repel the leeches was noted using stop watch. Cinnamon oil was found to be most effective among the evaluated essential oils.

Keywords leech, biorepellant, essential oil

1. Introduction

Leeches are segmented worms of phylum annelida. Leeches have two suckers, one at each end. Most leeches live in freshwater environments; some species are seen in marine as well as terrestrial environments. Leeches are haematophagus, feeding on vertebrate blood and invertebrate haemolymph. *Hirudo medicinalis* has been used in medicine to remove blood from patients.

Repellent is a substance applied to skin, clothing or other surfaces which discourages insects or other organisms from landing or climbing on that surface. Repellents are of two types- synthetic and natural. Synthetic repellents are more effective than natural repellents. They show repellence longer than natural repellents. Their disadvantages include rashes, swelling and eye irritation. DEET (N,N-diethyl-m-toluamide) may even cause cancer and defect in child birth. The chemicalspersist in the soil and reach water sources when they are washed off from the body surface during rains or bathing.

Natural repellents have advantages such as reduced irritation, nontoxic, eco-friendly, safer on sensitive skin. Their disadvantage is that they need more application than synthetic repellents.

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People working in leech infested areas constantly face the problem of leech bites, skin injury, infections and ulceration. They resort to use of common salt to ward off the leeches.

Oils of *Callistemon rigidus* (bottle brush), *Zanthoxylum armatum*, *Azadirachta indica* (neem) and synthetic repellent N, N- di-ethyl meta toluamide (DEET) and dimethyl phthalate(DMP) were evaluated for leech repellence properties and persistence on cloth by Nath *et al* (2002). The efficiency of certain oils against land leeches was investigated by Nath *et al* (1986). The persistence of leech repellent was studied by Nath *et al (*1993). Field evaluation of three repellent against mosquitoes, black flies and land leeches was conducted by Kumar *et al* (1984). Dixit and Khalsa (1967) evaluated repellents against land leeches.

This study evaluated the activity of selected essential oils against the leech *Haemadipsa*, as an eco- friendly alternative to chemical repellents.

1.1.Study Area

The area selected for the study was Ponkunnam of Kottayam district. It is located in the eastern high ranges of Kerala. People working in plantations and locals face the problem of leech bite.

1.2.Preparation of Essential oils as repellants for Leech

The essential oils selected for this study were -basil oil, ginger oil, vetiver oil, turmeric oil, lemongrass oil, cinnamon oil, clove bud oil and coconut oil. 5 ml of each essential oil was mixed with an equal volume of coconut oil. This extract was stored in labelled bottles

1.3.Field Observation

The trials were conducted during early hours of the day (7 am-10 am), when the leeches were more active. Eight volunteers were taken to the leech infested area. An essential oil was applied on one foot of a volunteer and he was allowed to walk through the leech infested area. The time of repellence was recorded using a stopwatch. The same procedure was repeated using other essential oils.

When the leech attached to the foot on which the oil is applied, it indicated that the oil did not have repellent properties. If the leech did not attach to the foot on which the oil is applied, it indicated that the oil had repellence against leeches.

The data obtained was analysed to evaluate the relative efficiency of essential oils as bio repellents for leeches

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2. Results and Discussion

The results of field trials revealed that five of the selected essential oils show repellency against leeches (Table 1).Cinnamon oil, clove bud oil show high repellency. They give excellent protection from leeches.

Sl no	Name of oil	Applied Time (min)	Repellency +(present) -(absent)
1	Coconut oil	5	-
2	Vetiver oil	5	+
3	Cinnamon oil	15	+
4	Basil oil	5	-
5	Turmeric oil	5	-
6	Ginger oil	5	+
	Clove bud oil	15	+
8	Lemon grass oil	8	+

Table 1 Repellency of leeches using essential oils

The decreasing order of repellence is cinnamon oil > clove bud oil > ginger oil > vetiver oil > basil oil > lemon grass oil > turmeric oil > coconut oil.

The results of the study indicate that cinnamon oil and clove oil are highly repellent (Figure 1) as well as toxic to leeches. When leech come in contact with these oils they become dead. It can be used to prevent leech bite on the field.

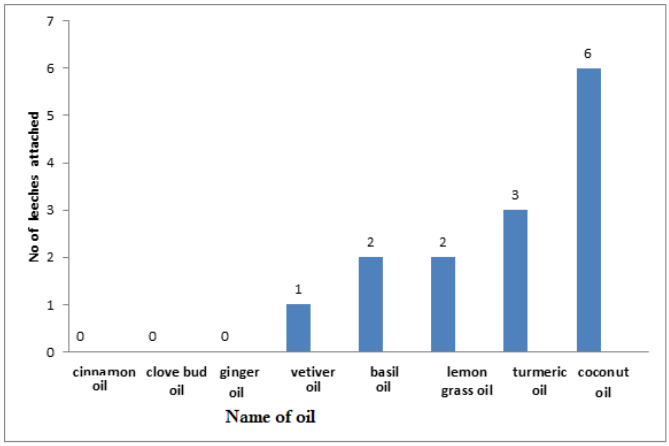

Fig. 1 Number of leeches attached to foot in the trials using essential oils

Conclusions

In this study, out of the eight essential oils, cinnamon oil and clove bud oil were found to be highly effective as repellents against *Haemadipsa*. Ginger oil, vetiver oil also show repellence. The essential oils can be more useful to the tribes, tourists,

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people who live in leech affected areas, forest officers etc. They spent major part of their time in the forests and have high chance of the leech bite.

The essential oils are produced using various plants. They are required in small quantities. Many essential oils can be used as repellents for land leeches when mixed with coconut oil. They can be prepared easily. This natural leech repellent can be recommend to people who working in leech infested area. They are safer alternatives to chemical leech repellants. They are retained on the skin for longer time than salt. Further studies are to be done in preparing the extract on a commercial basis for easy application (cream/lotion).

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Article 2

Production and Partial Purification of Tannase by Utilization of Cashew Husk as Economic Raw Material

Shajitha G.and Nisha M.K

Abstract

Tannase is an enzyme which catalyses the hydrolysis of tannic acid.The present investigation deals with production and partial purification of tannase enzyme from Aspergillus oryzae.The stain was isolated from cashew nut husk decomposed soil.Optimization of culture condition for tannase enzyme were studied and the optimum culture conditions determined were tannase activity enhanced on 9^{th} day of incubation,3% of substrate concentration ,30^{o}C temperature and the pH 5.0. Tannase was partially purified2.48 fold with a specific activity 4.148 U/ Mg protein and a recovery yield of 64.66 %. The enzyme produced from A. oryzae (crude, ammonium sulphate precipitated and dialysed) was tested for their antifungal and antibacterial activity by agar well method and agar disc method. It was observed from the results, the dialysate tannase produced from A. oryzae showed maximum zone of inhibition of 56.0 mm in antifungal activity against the phytopathogenic fungi, Aspergillus niger and highest inhibitory effect of 37.0mm in antibacterial activity against Streptococcus test bacteria by agar well method.

Keywords Tannase, Partial Purification, Cashew husk, Antifungal activity, Antibacterial activity.

1. Introduction

Research on usage of agro wastes as economical raw material for the production of industrially important products become more important these days. More than 40% of the waste from agricultural and food sources, agro industrial waste materials are generally considered as the substrate for the process of enzyme production. This boosts up high economic returns in many industrial practices, as agro waste provides alternative substrate, also restricts the amount of organic wastes reaching the landfills and helps to get rid of the pollution problems of waste disposal. The cashew nut coat causes an environmental problem for the producing regions, due to the high production of nuts and the manufacturing plants have no use for this residue, except for utilizing it to power boilers. The film, or coat, represents around 3% of the nut's weight, it is rich in tannins (Lima, 2009), and the nut, the edible part, formed by two ivory cotyledons, represent approximately 28% to 30% of its weight. Cashew testa or husk is the outer skin of cashew kernel which contains about 25% of tannin material. It is a low cost agro-waste substrate plenty in the southern part of India. The high

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hydrolysable tannin content in cashew husk can be effectively utilized for economical production of industrially important tannase enzyme. Agro-debris and forest products are generally considered the good source of tannin-rich substrate.

Tannins are considered to be secondary metabolic products from plants because they play no direct role in the plant metabolism. Tannins are the second most abundant group of plant phenolics, after lignin and the fourth most abundant plant constituents after cellulose, hemicellulose and lignin. One of the major feature of tannins is to form strong complexes with protein and other macromolecules such as starch, cellulose, and minerals. Tannins are known for their antimicrobial property and are resistant against microbes to protect plant bodies. Tannase catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid and gallic acid esters (Belmares*et al*., 2004). Tannase is broadly used industrial sector, food and in drug store. The main applications of this enzyme are in the production of gallic acid which is a pharmaceutically important compound, needed for production of trimethoprim and propyl gallate (Chavez-gonzalez*et al*., 2012), in the preparation of cosmetics, hair productions, lubricants, adhesives, dyes and photographic film development (Aguilar *et al*., 2007). Realizing the importance of the enzyme tannase, efforts were made to find a suitable micro-organism, which may produce higher amounts of tannase. Various fungal species have been represented to produce tannase, including *Aspergillus aculeatus, A. aureus, A. flavus, A. foetidus, A. japonicas, A. niger, A. oryzae, Aureobasidium pullulans, Fusariumsolani, F. subglutinans, Paecilomyces variotii, Penicillium atramentosum; P.chrysogenum, P. variable, and R. oryzae A .ruber , A. fumigatus*(Belur and Mugeraya, 2011). Because of the expanding modern utilization of tannase, new explores are important to discover new microbial sources with high creation of tannase.. Thus the present study aims in isolation, production, partial purification and antifungal activity of tannase obtained from *Aspergillus oryzae.*

2. Material and Methods

2.1. Isolation, screening and identification of native mycoflora from Cashew-nut husk decomposed soil

Soil samples from the disposal yard of Cashew-nut industry, Varadarajanpet, Ariyalur District, Tamil Nadu were collected and serially diluted and plated on potato dextrose agar medium and incubated for 5 days at 30°C. The fungal colonies developed were isolated, sub-cultured repeatedly for pure culture and maintained on agar slants. The fungal isolates were identified based on their morphology, mycelia structure and spore formation (Barron, 1968; Ellis, 1976 and Domsche and Gams, 1972). Primary screening for highest tannase producers was carried out using Congo red solution. The plates were point inoculated with the isolate with one percent tannic acid agar plates and incubated at 28^{o}C. The diameter of clear zones (including the colonies diameters) formed as a result of the hydrolysis of tannic acid around the

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fungal colonies were measured. The fungal strain showing largest zone of decolorization was selected for enzyme production.

2.2. Isolation of fungal genomic DNA and Analysis of 18S rRNA Gene Sequence

Five days culture of fungal mycelium was taken for DNA isolation. Genomic DNA of the fungal isolate was extracted using CTAB DNA extraction procedure (Kumar *et al.*, 2014) and employed as templates in PCR reaction using the universal 18S rRNA gene primers 18S-1 (5´-CCTGGTTGATCCTGCCAGTA-3´) and 18S-2 (5´-GCTTGATCCTTCTGCAGGTT-3´). The amplified 18S rRNA genes of each strain were sequenced on an automated DNA sequence with forward and reverse primers. For gaining the complete sequences of the 18s rRNA genes, EditSeq and SeqMan software were employed. Sequence similarities for the complete sequences for the 18s rRNA genes of different fungal strains were determined via Blast analysis available in the NCBI database. The phylogenetic tree was constructed by means of the MEGA - 7 software using neighbor joining meth.

2.3. Inoculum Preparation and Production of Tannase

For preparation of inoculum, 10 ml of sterilized distilled water supplemented with 0.1% Tween-80 was added to agar slant culture and the volume of 1 ml of spore suspension was used as the inoculum. Enzyme production was performed in 250 ml Erlenmeyer flasks containing 100 ml tannin containing basal liquid medium at pH 5.0 with 1% (v/v) inoculum and incubated at 30°C. Tannin containing Basal liquid medium at pH 5.0 was used throughout the study. The substrate was added in the medium at different concentrations (1%, 2%, 3%,and 4%) and enzyme activity was analyzed on the 5^{th}, 7^{th}, 9^{th}, and 11^{th} days of incubation. The fermentation medium was filtered through Whatman No.1 filter paper. The obtained filtrate was used for extracellular tannase determination and Intracellular tannase was extracted from the mycelium.

2.4. Estimation of Tannase Activity (Mondal and Pati, 2000)

The reaction mixture contained 0.3ml of tannic acid (1% in 0.2 M citrate buffer, pH 5.0), 0.1 ml of enzyme solution and incubated at 40°C for 30 minutes. The reaction was terminated at 0°C by the addition of 2ml of BSA (mg/ml), which precipitates the remaining tannic acid. A control reaction was also done side by side with the heat-denatured enzyme. The tubes were then centrifuged at 5,000x g, for 10min. The precipitate was liquefied in 2ml of SDS-triethanolamine 1% w/v, (SDS in 5% v/v of triethanolamine) solution and the absorbency was determined at 550nm after the addition of 1ml of $FeCl_3$ (0.13 M). A standard graph was prepared with gallic acid in the concentration range of 50µg/ml. The protein concentration was estimated according to the method Lowry *et al.* (1951).

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2.5. Optimization of culture conditions for tannase production.

Agro waste cashewnut husk collected from Cashew-nut industry, Varadarajanpet, Ariyalur District, Tamil Nadu were washed with distilled water properly. After cutting they were shade dried and powdered in good order.To assess the enhancement of enzyme production by *Aspergillus oryzae*, different concentrations of 1%, 2% ,3% and 4% Cashew-nut husk as substrate was supplemented in the fermentation medium at different temperatures (25°C, 30°C, 35°C and 40°C),and at different pH (4.5, 5.0, 5.5, and 6.0). The extra and intracellular tannase activities were analyzed at an interval of 2 days starting from 5th day to 11th day.

2.6. Partial Purification of the enzyme

Ammonium Sulphate precipitation and Dialysis

Partial purification of the crude enzyme was done by fractional precipitation of ammonium sulphate (Simpson, 2004). The enzyme activity and protein content of the fractions were measured. The fraction showing highest specific activity was used for further purification. The precipitate obtained after ammonium sulphate precipitation was further dialyzed against the 0.1 M citrate phosphate buffer (pH 5.0) in order to remove the ammonium sulphate from the precipitate. Dialysis was carried out for 24 hrs with several changes of 0.1 M citrate phosphate buffer at a pH 0f 5.0. (Roe,2001). The dialysate was analyzed for protein content and enzyme activity. Active fractions were freeze dried and stored at 4° C for further studies.

Antimicrobial activity

Cultures of *Aspergillus niger* and *A. flavus*kept in the fungal culture bank of Department of Botany, Avinashilingam University, Coimbatore were taken and maintained as subculture in slants. The antifungal activity of the enzyme is determined by the following agar Disc Diffusion Method (Barry and Thornberry 1991) andAgar Well Diffusion Method (Gobdi and Irobi, 1992). After incubation the diameter of the inhibition zone was measured using Fluconazole as a standard for comparison of antifungal activity.For the Antibacterial activity test bacteria of *Pseudomonas* and *Streptococcus* were collected from Bacterial culture bank, Department of Biochemistry and Biotechnology, Avinashilingam University Coimbatore and maintained as subculture in slants. The antibacterial activity of the enzyme is determined by the Disc Diffusion Method (Maruzzella and Henry, 1958) and Agar Well Diffusion Method (Perez *et al.,* 1990).The plates were incubated at 30˚C for 24 hours. After 24 hours the zone of inhibition around each disc was measured and the diameter was recorded using Streptomycin as the positive control and Citrate buffer used as solvent for the extraction as negative control for comparison.

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3. Results and Discussion

3.1. Isolation and Screening of tannolytic fungi (Hydrolyzing zone)

The native mycoflora isolated in large numbers from cashewnut husk decomposed soil samples were screened based on the formation of clear zones around the microbial colonies. Only 8 fungal strains (*Aspergillus oryzae, Aspergillus flavus, Trichoderma viride, Rhizopus spp.,Aspergillus nidulans, Trichoderma harzianum, Cladosporium batiana, and Aspergillus wentii*) showed positive reactions to qualitative test of tannase production as indicated by the clear zone formation in tannic acid agar media plates (Table -1). The appearance of the clear zone around the colony after the addition of congo red solution was a strong evidence for the secretion of tannase. Diameters of the colony and the clear zones were measured.Among these 8 fungal isolates a significantly highest hydrolyzing zone (clear zone) of 51 mm out of colony diameter of 56 mm was shown by *Aspergillus oryzae*. Since, *Aspergillus oryzae* showed remarkably prominent clear zone, it was selected as a candidate for the enzyme study. Filamentous fungi of the *Aspergillus* and *Pencillium* genus have been widely used for tannase production (Belur and Mugeraya 2011) Shajitha and Nisha (2018) observed significantly highest hydrolyzing zone on tannic acid agar plate by *Trichoderma viride*(54 mm).

S.No	Fungal stain	Colony ameter(mm)	Hydrolytic one(mm)
1.	*Aspergillus oryzae*	56.0	51.0
2.	*Rhizopus spp.*	53.0	49.0
3.	*Trichodermaviride*	42.0	39.0
4.	*Cladosporiumbatian*	25.0	23.0
5.	*A. nidulans*	25.0	22.0
6.	*A. flavus*	24.0	22.0
7.	*A. wentii*	25.0	21.0
8.	*T. harzianum*	21.0	18.0

Table - 1: The Diameter of the Colony and Hydrolytic Zone

3.2. Molecular characterization of *Aspergillus oryzae*

The PCR amplification of 18s rRNA gene, using the universal primers ITS1 and ITS4 resulted in a fragment which was approximately 650 bp. The same universal primer resulted in a 600 bp fragment in 108 *Aspergillus sp*. isolated from coffee bean to check the organisms involved in contamination of coffee bean by Magnani*et al*. (2005). The amplified product was sequenced and deposited in NCBI (Accession Number: MH191254.1), which was around 559 bp.BLAST analysis showed identical 18s rRNA sequences from *Aspergillus flavus* and *Sarocladium strictum* were found to be 99% identical to the sequence that we have done from *A. oryzae* isolated from the decomposed soil of cashewnut husk. Genomic sequence of a functional tannase

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gene has been described by *A. oryzae* and *A. niger* (Kazemi*et al.*, 2011) and a partial sequence for tannase enzyme was done by Kamal *et al.* (2016).

Gen bank formats of 18s rRNA of *Aspergillus oryzae* isolated from Cashewnut husk decomposed soil

LOCUS MH191254 559 bp DNA linear PLN 17-APR-2018

DEFINITION Aspergillus oryzae isolate Husk Decomposed Soil internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

ACCESSION MH191254

VERSION MH191254.1

KEYWORDS .

SOURCE Aspergillus oryzae

ORGANISM Aspergillus oryzae

Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina;Eurotiomycetes;Eurotiomycetidae;Eurotiales;

Aspergillaceae; Aspergillus.

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JOURNAL Submitted (12-APR-2018) Department of Botany, Avinashilingam Institute for Home Science and Higher Education for Women, Mettupalayam Road, Coimbatore, Tamil Nadu 6410 043, India

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Sequencing Technology :: Sanger dideoxy sequencing

##Assembly-Data-END##

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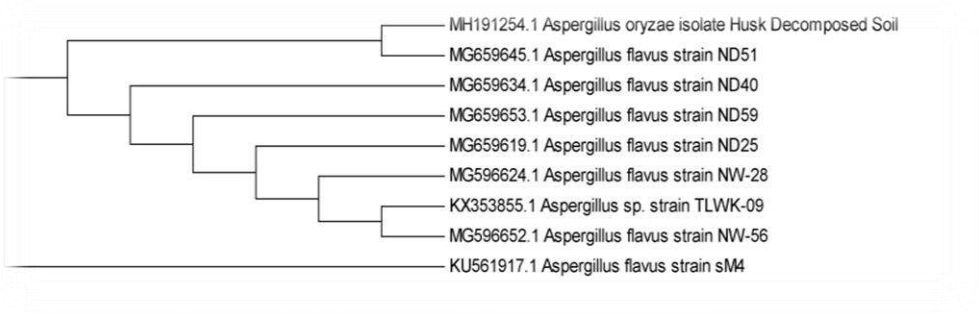

Phylogenetic analysis results for 18s rRNA of *Aspergillus oryzae*

Phylogenetic analysis by FASTA shows *Aspergillus flavus* strain ND51 18s to be closer than the other *A. flavus* strain

3.3. Optimization of culture conditions for tannase production

Effect of cashew nut husk as substrate on enzyme production (Table-2)

Among the different substrate concentrations (1, 2, 3 and 4%) the highest tannase activity was recorded in 3% (0.977± 0.003Umg^{-1} protein) on the 9th day of incubation of *A. oryzae* compared to control (0.263± 0.003 Umg^{-1} proteins) at an intracellular level. At an extracellular level tannase activity was much pronounced in 3% (0.965± 0.005 Umg^{-1} protein) followed by 2% (0.752 ± 0.002Umg^{-1} protein) compared to control (Table - 2). The present result is in accordance with the view of Kapoor and Iqbal, (2012) who reported that the enzyme showed appreciable activity with pomegranate rind (97%) as substrate.

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Cashewnut husk as Substrate	Concentration	Days			
		5	7	9	11
Intracellular	Control	0.104 ± 0.004	0.185 ± 0.003	0.263 ± 0.003	0.037 ± 0.003
	1%	0.215 ± 0.005	0.242 ± 0.015	0.371 ± 0.002	0.198 ± 0.003
	2%	0.435 ± 0.005	0.702 ± 0.003	0.925 ± 0.005	0.560 ± 0.002
	3%	0.861 ± 0.005	0.881 ± 0.002	0.977 ± 0.003	0.585 ± 0.004
	4%	0.145 ± 0.003	0.149 ± 0.006	0.333 ± 0.005	0.101 ± 0.002
Extracellular	Control	0.154 ± 0.004	0.202 ± 0.002	0.420 ± 0.003	0.599 ± 0.002
	1%	0.309 ± 0.002	0.318 ± 0.003	0.457 ± 0.003	0.388 ± 0.002
	2%	0.738 ± 0.054	0.388± 0.003	0.752 ± 0.002	0.615 ± 0.003
	3%	0.348 ± 0.003	0.705 ± 0.004	0.965 ± 0.005	0.298 ± 0.005
	4%	0.205 ± 0.003	0.300 ± 0.002	0563 ± 0.003	0.197 ± 0.513
SEd CD (p<0.05)		**0.067** **0.132**			

Results are the mean ± standard deviation of triplicates
Umg^{-1} = µ molgallic acid released min^{-1} mg^{-1} protein

Table - 2 Tannase activity (Umg^{-1} protein) of *Aspergillus oryzae* on cashewnut husk waste as substrate

3.4. Effect of temperature on enzyme production

The fungal isolates cultivated in the production medium was incubated at various temperature(25°C, 30°C, 35°C and 40°C) to observe the optimum temperature of tannase production. An enzyme production by *A. oryzae* was found to be increased from 0.584 ± 0.005 to 1.347± 0.002 at an intracellular level and 0.599 ± 0.002 to 0.781± 0.010Umg^{-1}protein at an extracellular level from 25°C to 30°C and peaked at 30°C on the 9th day of incubation. Further increase in the temperature showed a decreasing trend (Table -3). The present result coincides with the results of Malgireddy and Nimma (2015) who observed maximum tannase production (46.2 U/mg) at 30°C by *Aspergillus terreus*. Lal and Gardner *et al.* (2012) reported the maximum tannase activity of 195.0 U/ml at an incubation temperature of 30°C by *Aspergillus niger*.

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Cashewnut husk as substrate	Days	Temperature (°C)			
		25	30	35	40
Intracellular	Control	121± 0.005	411± 0.002	432± 0.005	304± 0.002
	5	248± 0.001	986± 0.001	832± 0.002	402± 0.001
	7	314± 0.003	001± 0.003	783± 0.004	413± 0.003
	9	585 ± 0.005	347 ± 0.002	671 ± 0.002	428 ± 0.002
	11	521 ±0.003	920 ± 0.003	918 ± 0.003	319 ± 0.003
Extracellular	Control	234 ± 0.002	461 ± 0.002	163 ± 0.002	398 ± 0.002
	5	276± 0.003	532± 0.003	245± 0.003	403± 0.002
	7	363± 0.004	634± 0.001	543± 0.004	423± 0.001
	9	599 ± 0.002	781 ± 0.010	611 ± 0.002	499 ± 0.003
	11	432± 0.001	666± 0.002	521± 0.001	409± 0.004
SEd		**0.067**			
CD ($p<0.05$)		**132**			

Results are the mean ± standard deviation of triplicates
Umg^{-1} = µ molgallic acid released min^{-1} mg^{-1} protein

Table 3 Tannase activity (Umg^{-1} protein) of *Aspergillusoryzae*on different temperature

3.5. Effect of pH on enzyme production (Table - 4)

Tannase from *A. oryzae* showed an enhanced activity from 0.778 ± 0.002 to 1.193 ±0.003 Umg^{-1}protein at an intracellular level and from 0.470 ± 0.003 Umg^{-1} protein to 1.110 ± 0.002 Umg^{-1}protein at an extracellular level upto a pH 5.0 and after that its activity decreased. This is because of the salt formation of tannic acid at high pH.Hamdy and Fawzy (2012) reported the maximum tannase production by *A. niger*(96.11, 114.76, 106.8 U 50 ml-1 for LSF, SSF, SlSF respectively) was optimally recorded at an initial pH of 5.0 of the fermentation medium.

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Cashewnut husk as	Concentration	pH			
		4.5	5.0	5.5	6.0
Intracellular	Control	0.601±0.002	0.701±0.002	0.619±0.002	0.200±0.00
	3%	0.778±0.002	1.193±0.003	0.881±0.003	0.411±0.00
Extracellular	Control	0.308±0.003	0.708±0.003	0.499±0.003	0.530±0.00
	3%	0.470±0.003	1.110±0.002	0.741±00.003	0.709±0.00
SEd CD (p<0.05)		**0.002** **0.004**			

Results are the mean ± standard deviation of triplicates

Umg^{-1} = µ molgallic acid released min^{-1} mg^{-1} protein

Table4:Tannase activity (Umg^{-1} protein) of *Aspergillusoryzae* on different pH at 3% cashewnut husk as a substrate at 30°C

3.6. Purification of Tannase

Among the various ammonium sulphate precipitated samples, the highest tannase activity of 6.981±0.002 U and specific activity of 3.876±0.004Umg^{-1} with purification fold of 2.322 was noted in 60-80 percent ammonium sulphate sample. Similar result was obtained by Swetha *et al.* (2007) who reported the crude enzyme activity of 0.81u/ml of protein and by using ammonium sulphate the activity increased up to 1.03 81U/ml of protein.From the Table 5, it is clear that there was almost a 2% decrease in total tannase activity 4.547±0.003U when compared with the ammonium sulphate precipitated sample 6.981±0.002U where as an increase in the specific activity (4.148±0.001Umg^{-1}) was observed in dialyzed sample than the ammonium sulphate precipitated sample (3.876±0.004Umg^{-1}). The increase in specific activity ensures the progress in purification of the tannase. The results coincides with the result of Anitha and Arun Kumar (2013) who observed the higher specific activity of 11.41 with the purification fold of 5.46 where as specific activity of the diaylsate showed 77.6 with the purification fold of 6.8 to the tannase enzyme obtained from *Mucor*sp

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S.No	Samples	Protein content (mg)	Tannase activity (U)	Specific activity (U/mg)	Purification fold	Recovery %
1.	**Crude extract**	4.212	7.032±0.003	1.669± 0.022	1	100
2.	**$(NH_4)_2SO_4$ precipitated Sample (60-80%)**	1.801	6.981±0.002	3.876±0.004	2.322	99.27
3.	**Dialyzed**	1.096	4.547 ±0.003	4.148 ±0.001	2.485	64.66

Results are the mean ± standard deviation of triplicates

Umg^{-1} = μ molgallic acid released min^{-1} mg^{-1} protein

Table - 5 the partial purification of tannase obtained from *Aspergillusoryzae*

3.7. Screening of the partially purified enzyme for Antimicrobial activity

The enzyme produced from *A .oryzae*(crude, ammonium sulphate precipitated (60-80%) and dialysate) were tested for their antimicrobial activity against the test pathogenic fungus (*Aspergillus flavus* and *A. niger*) and bacteria (*Streptococcus* and *Pseudomonas)* by agar well and disc diffusion method.

3.8. Antifungal Activity

When compared to agar well and disc diffusion method, maximum inhibitory effect against phytopathogenic fungi was found to be in agar well method and is summarized in Table 6. The results of the study showed that Dialysate tannase was found to possess better antifungal activity by agar well method with the highest zone of inhibition of 56.0 and 51.0mm against phytopathogenic fungi *Aspergillus niger* and *A.flavus* respectively. Muslim *et al* (2017) reported that the ceftazidime at a concentration of 128μg/ml gave higher inhibition zone (31 mm) against *Psedomonas aeruginosa*, while the concentration 16μg/ml showed lower inhibition zone (24 mm)

Pathogenicfungalstrains	Antifungal activity	Zoneofinhibition(mm)				
		Crude	Ammonium sulphate precipitated	dialysate	Positive control (Fluconazole)	NegativeControl(CitrateBuffer)
Aspergillus flavus	**Agar well diffusion**	16.0	32.0	51.0	27.0	11.0
Aspergillus niger		20.0	43.0	56.0	31.0	15.0
Aspergillus flavus	**Agar Disc diffusion**	21.0	11.0	34.0	31.0	13.0
Aspergillus niger		0.0	12.0	23.0	21.0	4.0

Table-6 Inhibition zone of tannase against phytopathogenicfungiby *Aspergillus oryzae*

3.9. Antibacterial activity

From the results of the study summarized in Table 7 it was observed that in agar well diffusion method, the dialyzed tannase obtained from *A. oryzae* showed the highest inhibitory effect of 37.0mm followed by ammonium sulphate

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precipitated tannase (26.0mm) against *Streptococcus* test bacteria. In the disc diffusion method the dialyzed enzyme produced from *A. oryzae* showed maximum inhibition in *Pseudomonas* (33.0mm) and *Streptococcus* (29.0mm). Doss et al. (2009) watched that tannins shown antibacterial activity against Staphylococcus aureus, Streptococcus pyrogens, Salmonella typhi, Pseudomonas aeruginosa, Proteus vulgarisand Escherichia coli and reported that S. aureus was the foremost safe to tannins confined from the plant fabric taken after by Streptococcus pyrogens, Salmonella typhi, Escherichia coli, Proteus vulgaris and Pseudomonas aeruginosa. Minimal inhibitory concentration of the tannins ranges b/w 1.0 and 2.0 mg/ml, 1.5 and 2.0 mg/m is the lowest bactericidal concentration.

Pathogenic bacterial strain	Antibacterial activity	Zone of inhibition(mm)				
		Crude	Ammonium sulphate precipitated	Dialysate	Positive control (Streptomycin)	Negative Control (CitrateBuffer)
Streptococcus	**Agar well diffusion**	20.0	26.0	37.0	22.0	13.0
Pseudomonas		13.0	21.0	30.0	25.0	7.0
Streptococcus	**Agar Disc diffusion**	13.0	15.0	29.0	21.0	11.0
Pseudomonas		6.0	25.0	33.0	26.0	14.0

Table-7 Inhibition zone of tannase against phytopathogenicbacteria by *Aspergillus oryzae*

Conclusions

Tannase has now been extensively used in variety of biochemical industries. The selected fungi *Aspergillus oryzae* used in this study is able to synthesize high amounts of tannase through submerged fermentation. Exploitation of this agro waste (cashew-nut husk) could be a source of cheaper substrate for industrial production of microbial tannase. The present study of enzyme produced from *A. oryzae* (crude, ammonium sulphate precipitated (60-80%) and dialysate) showed strong antifungal activity against Pathogenic fungi *Aspergillus flavus* and *A. niger*and antibacterial activity against *Streptococcus* and *Pseudomonas.* Thus, the present investigation suggests that cashew-nut husk as substrate can be one of the best and, cost effective alternatives to the costly pure tannic acid production.

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Article 3

Mechanistic insight of Protein aggregation kinetics and limiting factor

Ajeet Singh, Garima Srivastava, Ragini Sikarwar and Ruchita Srivastava

Abstract

The phenomena of protein aggregation have a significant impact in a number of diverse fields ranging from medicine to material science to nanotechnology. The protein aggregation may occur at any stage of its lifespan; aggregates may be manifested into insoluble fibrils, amorphous aggregates and soluble oligomers based on their morphology. Recently, this field has gained an overwhelming momentum due to its contribution in several devastating human neurodegenerative disorders, for example, Alzheimer's disease, Parkinson's disease and prion mediated diseases. In addition, the discovery of non-pathological protein aggregates *in vivo* or *in vitro* has further directed aggregation research towards mechanistic analysis and its control. Hence, despite the huge complexity of the aggregation mechanism, the process itself appears intrinsic properties of polypeptides chains. This particular review summarizes research highlights associated with aggregation kinetics and certain aggregation associated neurodegenerative diseases found in humans. Protein aggregation is a sensitive phenomenon where different type of factors play crucial role. In this review paper, we have briefly discussed these factors along with aggregation kinetics.

Keywords Aggregation, Nucleation-Dependent Polymerization (NDP), Isodesmic Polymerization (IDP), Fourier Transform Infrared Spectroscopy (FTIR).

1. Introduction

Proteins are synthesized into Endoplasmic Reticulum (ER) and transported to respective organelles through post or co-translational protein trafficking for further functional activities. A protein requires a proper folded conformation known as nativeconformation for catalyzing any specific biochemical reaction under physiological conditions. Therefore, diverse regulatory machineries had evolved for proper regulation of the protein folding inside the cellular system and to minimize the protein-misfolding rate. There are many biological factors like the nature of protein, over expression of a specific protein, complex interactions with other biomolecules and the most apprehensive molecular crowding attribute in the protein misfolding, which finally direct the protein for aggregation.

Protein aggregation is a multi step process where a protein loses its native conformation and acquires a non-native conformation. Such structural modulation from a native to a non-native state may lead the corresponding protein with a loss of its functionality and may initiate the aggregation process. Insoluble fibrous aggregates are formed as an outcome of

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the aggregation process and are termed as ‘amyloid’. In around 1854, Rudolf Virchow, a German physician and scientist, coined the term amyloid to describe the iodine stained cerebral corpora amylacea that had an abnormal macroscopic appearance. Initially, these deposits were considered carbohydrates in nature until a high percentage of nitrogen were detected in 1859. Nevertheless, the inaccurately explanatory name, amyloid, is continued to be used for such insoluble fibrillar proteinaceous deposits (Cleland *et al*., 1999).

The formation of fibrils induces cellular stresses through an alteration of intracellular or synaptic transport along with other vital cell functions and finally directs the interruption of cellular physiology. Initially, the pathological side of these aggregates was not identified but Alois Alzheimer (1906) changed the whole aggregation paradigm towards pathological exploration.

Firstly, he observed the presence of abnormal deposits in brain tissue of a person who was suffering with a progressive memory loss. He continued the morphological analysis study of shrinkage brain tissue, later on Emil Kraepelin (1910) named this disorder as Alzheimer’s disease (Lansbury *et al.*, 2006).

Afterward, various aggregation-associated disorders were reported. The deposition of amyloid fibrils disrupts the cellular physiology and such physiological disruption causes a number of neurodegenerative as well as non-neurodegenerative disorders in the biological system. Amyloid fibrils are not restricted to any specific part of the cell. These pathological amyloid fibrils may accumulate in nucleus (cause Huntington’s disease), cytoplasmic inclusions (cause Parkinson’s disease), extracellular accumulations (cause Prion diseases) or both types of accumulation (cause Alzheimer’s disease by Aβ-amyloid and tau proteins). Presently, more than fifteen aggregation-associated disorders have been reported in humans and millions of people across the world are suffering from various neurodegenerative disorders.

Apart from aggregation mediated neurodegenerative diseases, the pharmaceutical sector is also under a rising anxiety from drug regulatory authorities to ensure the desired quality of synthesized drugs, as aggregation is a common consequence in the pharmaceutical sector. There are high possibilities for aggregation at almost every stage of the pharmaceutical processing, such as production, formulation, storage, and application. It also has been reported that aggregated drugs have a higher immunogenic response to patients that may have a series of deleterious consequences. The pathological as well as the non-pathological involvements of these aggregates have aggravated the scientists across the world for further exploration of the aggregation mechanism (Singh *et al.*, 2018).

1.2. Protein Folding Kinetics

The elucidation of the molecular structure of an amyloid fibril is essential to understand the aggregation mechanism as well as the biophysical interactions between monomers that facilitate amyloid formation under certain physiological conditions. As an initial, amyloid X-ray diffraction pattern was studied by William Astbury and he proposed a cross-beta (β) fibril

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pattern, a symphonic signature motif that was further accepted for amyloid state of proteins. Subsequently, Circular Dichorism (CD) and Fourier Transform Infrared Spectroscopy (FTIR) were also applied for beta (β)-sheet content measurement whereas Electron Paramagnetic Resonance (EPR) spectroscopyand quenched hydrogen/deuterium exchange coupled with mass spectrometry or two dimensional NMR were used for additional molecular insight about the amyloid local structures. Presently, it has been experimentally demonstrated that amyloid fibrils are rich in cross beta(β)-conformations; such cross beta(β)-rich composition of amyloid fibril provide the binding surfaces to Congo red/ Thioflavin-T dyes.

Initially, amyloid fibrils were reported *in vivo* into various neurodegenerative diseases, now there are a number of proteins that are not found to be associated with any disorder but still may form amyloid fibrils under appropriate *in vitro* conditions. These findings indicate that amyloid formation is a condition dependent intrinsic property of a polypeptide. Different models have been proposed to understand the aggregation mechanism and all models are based on beta (β)-sheet formation frequencies initiated by amino acid compositions. Subsequently, different statistical algorithms like TANGO, Aggrescan and Zyaggregator etc. have been developed to predict beta (β)-aggregating stretches in a protein for a particular set of physico-chemical parameters. Conclusively, the protein aggregation frequency can be predicted theoretically based on its primary sequence (Dill *et al*., 2012).

1.3. Types of Aggregation Kinetics

The protein aggregation mechanism is still elusive because of its multi step process. There are a number of models have been proposed to explain protein aggregation kinetics; the three most accepted models are, Nucleation-Dependent Polymerization (NDP), Isodesmic Polymerization (IDP) and Ring Chain or Opening Polymerization (RCP). Out of these three, the NDP model has been performed extensively in various biological systems.

In NDP also known as cooperative polymerization, polymers are multi stranded and have three distinctive platforms: critical concentration for nucleation, activation energy barrier and two uniformly distributed populations at equilibrium. The monomeric protein follows a structural alteration from its native conformation and finally adopts any amyloidogenic. Such conformational alteration from a native to a non-native state alters secondary interactions of the protein molecule. Subsequently, all these modulations of the monomeric protein alter its free energy, an essential and determining energy barrier for nucleation. Nucleation is the initial step of the aggregate formation that characteristically exists above the critical concentration range, which is condition dependent value for a protein and varies indifferent physiochemical aggregation conditions. Before the nucleus formation, kinetic parameters are not favorable for the aggregation progression and dimer or pseudo dimer could remain in the same phase for an indefinite time (Kurganov, 2005).

Characterization of such non-favored structures is also found to be challenging due to its dynamic multi component system and low experimental resolutions. These nuclei further form critical nucleus that accelerate the fibril formation. The time duration required for

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critical nucleus formation is considered as the lag period. The lag phase is considerably slow and the rate limiting step and may protect normal individuals from the amyloid fibril formation. The lag phase time is inversely proportional to the steepness of energy curve in initial step. The lag phase can be modulated either through modifications in aggregating conditions such as pH, temperature, shear rate and protein concentration etc. or by adding the preformed fibril called seeding. The seeding effect drastically reduces the lag time for any aggregation reaction. After critical nucleus formation, conditions become favorable for the protein aggregation with fast kinetics, which is known as the elongation phase. Any available monomer with amyloidogenic conformation rapidly participates in the fibril formation. At equilibrium in aggregation reaction pool, a finite monomeric amount would exist in equilibrium with aggregating polymer (Ferrone, 1999).

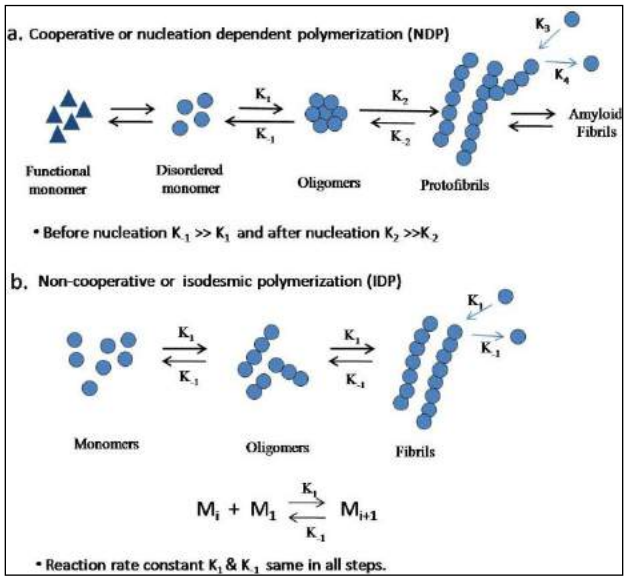

Fig 1. Diagrammatic representation of aggregation kinetic types.

In Non-cooperative or IDP, polymers are comparatively short, fragile, and single chained in morphology. The IDP kinetics is relatively faster than the NDP. Any kind of structural modulations is not present in IDP, therefore the secondary interactions between monomer-polymer are present with equalaffinity and are unaffected from the polymer length. The IDP rate is directly proportional to total protein concentration, in contrast to the NDP, where the monomeric concentration plateaus are above the critical concentration. Therefore, the amount of polymer gradually increases as the monomeric concentration is increased in the reaction pool. The IDP does not follow critical concentration or nucleus formation limitations and the kinetics can be considered similar to the elongation phase of the NDP model. The IDP can be initiated from any monomeric subunit without any conformational alteration and the addition of these monomers subsequently increases the thermodynamic stability of the system (Ahmad *et al*., 2017). The IDP model is summarized in Figure 1.

The third polymerization mechanism is known as Ring Chain mechanism; a special type of nucleated polymerization. If the monomeric subunit possesses a covalently closed ring type

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structure and forms a linear polymer, there are probabilities for such kind of polymerization. Above a critical concentration, reaction thermodynamic condition becomes favorable and subsequently polymer elongation rate increases at the terminal. Below the critical concentration, linear polymer ends react with each other and form a ring. Such special type of polymerization is rarely reported in the biological system but frequently present in the polymer chemistry, therefore most of the biological research groups have not intensified much research on this aggregation mechanism (Žerovnik, 2011).

1.4. Protein Aggregation Limiting Factor

Protein aggregation mechanism is still elusive because of its multi step process that facilitate by various factors. Diverse factors, both intrinsic and extrinsic can affect the protein aggregation mechanism. In intrinsic factors, genetical and structural factors are included; these factors play determining role in protein aggregation. Specific gene mutation, gene duplication, expression level of any specific gene and protein folding regulating machinery mutilation are most studied genetical factors that can considerably alter aggregation rate *in vitro*. Apart from genetical factors, structural factors like protein primary sequence, primary/secondary structure, protein charge, hydrophobicity and polar/non polar pattern also play determining role in protein aggregation.

Available experimental data shows that aggregation is an intrinsic property of any polypeptide and specific protein region, known as amyloidogenic region. These regions direct the protein to aggregation under appropriate conditions and havespecific characteristics with respect to hydrophobicity, polar/nonpolar pattern, charges, and secondary structure propensities that contribute significantly in aggregation. During the last decades, various computational algorithms such as TANGO, Aggrescan and Zyaggregator have been designed to predict these amyloidogenic regions through primary sequence. Most of these algorithms are based on specific characteristics of amyloidogenic regions and predict the amyloidogenic regions based on different physico-chemical parameters of amino acids that differ among the twenty amino acids.

Theoretical and experimental analysis has revealed that tryptophan, phenylalanine, cysteine and tyrosine have higher aggregation propensity while aspartic acid, lysine, arginine, and glutamic acid have the lowest aggregation propensity.

Apart from diverse physico-chemical properties, protein folding machinery had evolved various negative design strategies like incorporation of β-sheet breaker amino acid, absence of β-strands on the protein edge or positioned gatekeeper residues in close proximity to aggregating prone segments to diminish the higher aggregation propensities. Aggregation propensities are higher in β-rich structure as compare to other conformations, therefore incorporation of β-sheet breaker amino acid in β-rich region minimize the β-sheet propensities as well as aggregation. If β-strands present on the protein edge, such protein's aggregation propensities are higher as compare to α-helical containing structure on the protein edge.

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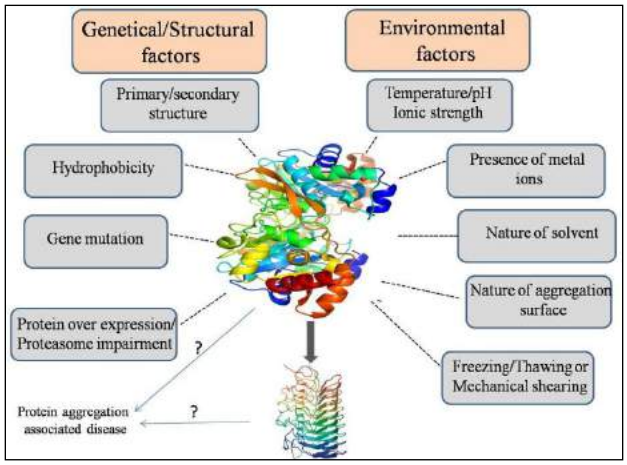

Fig.2 Protein aggregation limiting factor (genetical and environmental factors).

Therefore, folding machineries supports the presence of α-helical structure on the protein edge. Certain amino acid with low aggregation frequency, known as gatekeeper residues usually present in close proximity to amyloidogenic region. These gatekeeper residues considerably decrease the aggregation propensity through higher repulsive force (arginine, aspartic acid,glutamic acid, lysine), entropic penalty on aggregation because of their large side groups (arginine, lysine) or by β-sheet breaker property (proline) (Wang et al., 2010).

In the secondary structure, α-helices negatively modulate the aggregation rate while β-sheets positively support the aggregation rate. Proteins having more β-sheet structures tend to be more aggregative than more α-helix containing structures, due to higher dipole moment of α-helices. Net charge of a protein reduces the aggregation rate similar to α-helices. Hydrophobicity also affects protein aggregation propensity positively as more hydrophobic proteins more likely form aggregates. Further, aggregation studies in the presence of surfaces demonstrated that surface chemistry, relative amount of hydrophilic residues and local concentration of hydrophilic/hydrophobic residues in contiguous blocks of the mixed surfaces are correlated with the aggregation propensity.

Aggregation is a protein's intrinsic property that is strongly influenced by diverse extrinsic environmental factors like temperature, pH, ionic strength, shaking and shearing, protein concentration, nature of solvent, presence of metal ions, nature of contact surface and freezing/ thawing. These factors can destabilize the folded state and favor intermediates formation during the aggregation process. Temperature is most critical and influencing factor in aggregation among all these factors. Above a certain temperature, protein unfolding is usually followed by a fast aggregation rate. Higher temperature increases the protein aggregation rate by increasing both hydrophobic interactions and molecular collision frequencies. An increased temperature also may change the relative fraction of secondary structure and alter the aggregation frequencies. Lag time at a higher temperature may be eliminated or reduced as compared to low temperature lag time in the aggregation process.

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Another critical factor is solution pH, which can alter the type and distribution of the net charge on proteins and modulate the protein aggregation rate. Protein concentration is also another critical factor in protein aggregation that generally increases the aggregation rate at a higher concentration. In different protein aggregation studies, it has been observed that an increase in protein concentration can reduce lag time as a critical concentration is required to form initial nucleus for aggregation initiation. The effect of protein concentration on the aggregation kinetics is not universally followed by all proteins, for example increased concentration of human IFN-γ from 1 to 4 μM increases the lag time. The slower aggregation kinetics of human IFN-γ is because of an increase in dimer concentration, which is less effective for aggregation.

Presently, a number of human disorders are associated with inappropriate deposition of several protein aggregates *in vivo* and some of these disorders are recognized for a long time while some of them have been reported in the last two decades. Various research groups across the world have intensified their research to understand the structural and the pathogenic characteristics of these pathogenic aggregates that are still not fully understood. Hence, mechanistic studies of such more prominently present aggregation diseases like Alzheimer's disease, Parkinson's disease and Prion disease will give the overall idea about disease mechanism, its progression, as well as the therapeutics possibilities (Mahler *et al.*, 2009).

Conclusions

Protein aggregation is an intrinsic property of protein that is modulated by various factors. It also can be analyzed through various computer based tools like TANGO, AGGRESCAN & Zyaggregator etc. These tools predict beta (β)-aggregating stretches in a protein for a particular set of physico-chemical parameters. Protein aggregation kinetics is classified into three different types based on mechanistic differences. Protein aggregation kinetics also modulated through various structural and genetical factors.

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Article 4

Molecular Characterization of the Stress Tolerant Novel *Bacillus* Species from Fermented Rice Water

Subhashini Ramakrishnan, Abirami V, Akalya P, Dharun M, Shanmugam M and Vijayakumar M.

Abstract

Different traditional fermented foods are used by different communities during their rituals and customs since the olden days. Consuming such foods is healthy because of the beneficial organisms present. Only few such microbes were reported but still much need to be explored for its potential. The study screened the spore forming *Bacillus* sps.(MT186174)from fermented rice water using 16S rDNA sequencing.Investigation oftheGram-positive, spore forming and catalase positive organism tolerated the stomach pH range of 3, able to survive in the 0.3% (w/v) concentration of bile salts, survived efficiently at low and high temperature, lack antibiotic resistance genes. Also, the identified isolate possessed antimicrobial property against *Listeria monocytogenes*. These properties are crucial to explore the species further to produce useful products either in the food or feed industries.

Keywords Probiosis, Probiotics, *Bacillus* sps., Fermented rice, *Listeria monocytogenes*.

1. Introduction

Civilization of the human society leads to the development of fermented foods and beverages for the sustained nutrition and preservation of foods. Rice is one of the traditional foods in countries where people consume itas a staple food. The starch based foods are rich media for the growth of good microorganisms. The connection between the fermented foods, microorganisms and health benefits has emerged as a new science, Probiosis. Probiotics are live microorganisms consumed in the form of adequate dietary supplements/foodenhances the quality of host's life including the improvement of beneficial microbial flora. Though natural foods containing probiotics are notknown by all, many viable probiotic strains are available in the market in different forms such as powder, capsules, tablets, etc.Moreover, the market potential of probioticsis been hugely successful also in the developing countries.Identification of thenovel probiotic strains upshot the advent of products by food or feed industries in turn expandsthe Indian probiotic market where it also expected to reach $10 million by the year 2020 (Deshpande*et al.,* 2019).Many Gram positive bacteria serve as probiotics for human as well as for farm animals. Amongst, the spore forming*Bacillus*possessesseveral applications such as the production of enzymes, amino acids, antibiotics, preparation of fermented foods and as pest controlling agents (Nithya and Halami 2013). Hence, the present study focused on

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isolating and identifying such organism which is important in order to satisfy the increasing demand in the market which facilitates the development of new functional products.

2. Materials and Methods

2.1. Preparation of Rice Samples

Red and white rice samples (75g) were cooked with water for 30min. Fermentation was carried out in the closed clay pots by soaking 50 gof cooked and uncooked rice samples separately in the ratio of 1:3 (rice:water)at ambient temperature for 24 h. Then collected 10mlfrom soaked rice water, vortexed and diluted in sterile saline (0.85%) solution to obtain concentrations up to 10^{-4} dilutions. Aliquots (100µl) of diluted and undiluted samples were inoculated on to Petri plates containing De Man, Rogosa and Sharpe (MRS) agar and incubated anaerobically at 37°C.The morphological characteristics of isolates, Gram and endospore stainingwere determined.

2.2. Haemolytic Activity and Biochemical Analysis

Bacterial isolates evaluated forits haemolytic activity using blood agar and incubated at 37°C for 24 h. Biochemical characteristics of each isolate were analyzed with catalase, bileesculin hydrolysis, motility, oxidase, indole production, methyl red and Voges-Proskauer tests.

2.3. Carbohydrate Fermentation

The ability of the isolated organisms to ferment different sugars (dextrose, galactose, lactose, maltose and fructose) was determined in the suitable media (10ml) containing 1% of any sugar with an inverted Durham tube and inoculated with 50µl of overnight culture and incubated for 2days.

2.4. NACL and Temperature Tolerance

Tolerance to different concentrations (4% and 6.5%) of sodium chloride (NaCl) tested with 5ml of MRS broth containing bromocresol purple indicator followed by theinoculation with 50µl of each isolate and incubated for 4 days. Overnight culture (50µl) was transferred to the test tubes containing suitable medium. Added 2-3 drops of bromocresol purple indicator and incubated at different temperatures such as 15°C, 37°C, 45°C and 100 °C for 4 days. During incubation time, the microbial growth was observed by the change in the colour of the medium from purple to yellow (Mulaw*et al.*,2019).

2.5. Bile Salt and Acid Tolerance

100µl of overnight culture suspension was inoculated into tubes containing 20ml of MRS broth with 0.3% (w/v) Oxgall. The Optical Density(OD) valuewasmeasured during different time intervals at 620 nm (Mirlohi*et al.*,2009).1% (v/v) of overnight culture was inoculated into the prepared phosphate buffer saline (PBS) (8g NaCl, 0.2g KCl, 1.44g Na_2HPO_4, and 0.24g KH_2PO_4 in 1L distilled water) and adjusted to pH3 (with 1N HCL) followed by the incubation at 37°C for 3h.After which 1% (v/v) of cell suspension was inoculated into 10ml of MRS broth and incubated for 24 h. Cell growth was assessed by measuring optical density (OD) at 620nm (Shokryazdan*et al.*,2014).

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2.6. Antibiotic Susceptibility

Each isolate was measured for its antibiotic susceptibility by disc diffusion method against different antibiotics (Penicillin, Tetramycin, Cephalothin, Imipenem, Vancomycin, Erythromycin, Ciproflaxin, Trimethoprim, Rifampicin, Gentamycin and Kanomycin). Volume of 100µl of fresh culture was swabbed evenly over the surface of nutrient agar plates with sterile cotton swab. After drying, antibiotic discs were placed on the solidified agar surface, and left plates aside for 30 min at 4°C for the diffusion of antibiotics followed by the incubation for 24 to 48h.Zone of inhibition (ZOI)was measuredand expressed as susceptible (when zone is ≥ 21mm); intermediate (between 16-20mm), and resistance (≤15mm).

2.7. Anti-listerial Activity

Anti-listerial activity of the isolate was determined using agar-well diffusion method. The isolate was inoculated into MRS broth containing 1% glucose and incubated overnight at 37°C. Overnight active culture broth was centrifuged separately at 5000rpm for 10min at 4°C. Cell-free supernatant was collected as a crude extract for the antimicrobial study against the selected food-borne pathogen. Inoculated *Listeria monocytogenes* in brain heart infusion broth and incubated at 37°C for 24h. A volume of 100µl of the pathogen was swabbed evenly over the surface of nutrient agar plates with a sterile cotton swab.Sterile cork borer was used to cut uniform wells in the dried plates and filled with 100µlof culture-free filtrate was incubated at 37°C for 24 to 48h. Next, plates were observed for a zone of clearance (ZOC) around the well and zone was measured(Mulaw*et al.*, 2019).

2.8. Molecular Identification

Amplified DNA was submitted to XetraBiosolutions for sequencing.BLAST analysisof the identified sequence against known sequences available in the database resulted with hits. Based on the e-value and identity score, suitable hits were selected and performed evolutionary relationship analysis.Identified sequence was then submitted in the nucleotide database.

3. Results and Discussion

3.1. Morphological Characteristics of Isolates

Non-dairy based foods are gaining interest than dairy based due to different issues such as lactose in-tolerant, allergy, fat and cholesterol associated with milk products. Fermented rice is full of nutrition providing immense health benefits and produces very cooling effect in our body. Recent study by Borah*et al.*, (2019) identified the potential probiotic candidate from indigenous *Bacillus velezensis* strain DU14 from Apong, a traditionally fermented rice beer of Assam. Several probiotics probiotic were isolated from different sources but less number of molecular sequencesdeposited in sequence database.

In the present study,totally 17 different colonies were isolated and subcultured repeatedly in order to obtain the pure culture (2 each from cooked and uncooked fermented white rice (CFWR; UFWR); 7 from cooked and 6 from uncooked fermented red rice (CFRR;

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UFRR)). Out of 17 isolates, only 8 were Gram positive and these isolates subjected to Endospore staining revealed that CFRR1, CFRR5 and UFRR4 are only the spore formerswhich formed green colour during staining. Haemolytic activity of these three isolates showed the absence of lysis of cells indicating gamma haemolytic.

3.2. Biochemical Characterization

The spore forming and non-haemolytic isolates (CFRR1, CFRR5 and UFRR4) showed the presence of catalase which mediated the breakdown of hydrogen peroxide into oxygen and water evidenced by the rapid effervescence of oxygen bubbles.Also, isolates hydrolyzedesculin into glucose and esculetinby esculinase was confirmed by the blackening of the medium. Motility test showed that all isolates are non-motile and indicated the absence of oxidasewhich converts tryptophan to indole.These three isolates are having the ability to produce acetylmethylcarbinolwas indicated by the presence of pink colour in Voges-Proskauer test and in contrast showed the negative result for methyl red test.

3.3. Probiotic Properties

CFRR1 fermented dextrose and maltose whereas UFRR4 and CFRR5 fermented all sugars whereas the later isolate not fermented the lactose. All isolates tolerated both low and high concentration of NaCl was indicated by the change in the colour of the medium from purple to yellow.Temperature tolerance at different temperatures indicated CFRR5 and UFRR4 survived at both high and low temperatures whereas CFRR1 was able to grow only after three days possibly at 15ºC and one day after at 37ºC and 45ºCrespectively.

Tolerance to acidic and bile rich intestinal environment are essential for the probiotic culture to function effectively in the stressful environment.The pH of the stomach is anywhere between 1 to 3 and it can rise up to 4-5 after digestion. The ability of the isolated organisms in bile salt (0.3 %) and acidic conditionindicated that the growth rate of CFRR5 was high when compared to the other isolates and survived up to the maximum hour tested (6 h) (Fig. 1-3).ZulkhairiAmin *et al.,* (2020) isolated Gram and catalase positive isolates from stingless bee which survived in 0.3 % bile salt concentration for about only 3 h. In the preset study, the identifiedCFRR5 endured its ability in bile salt up to 6 h.

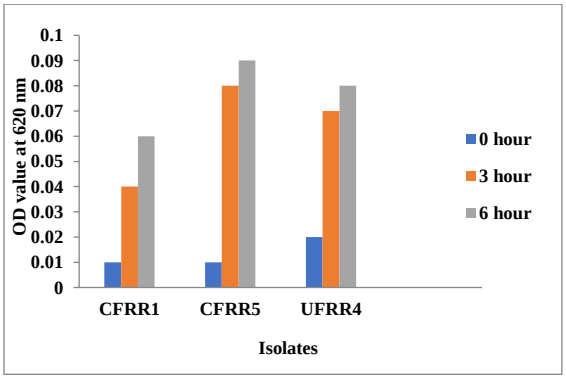

Fig. 1.Bile Salt Tolerance of Isolates

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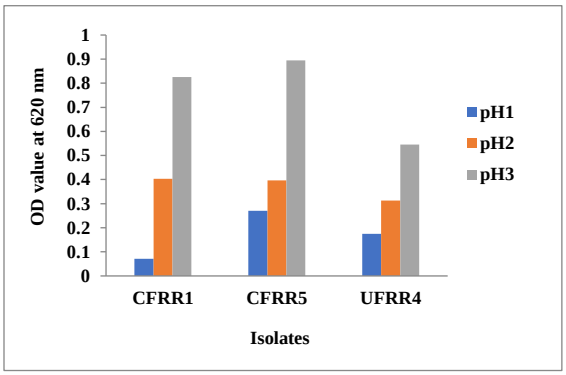

Fig. 2. Acid Tolerance of Isolates at Third Hour

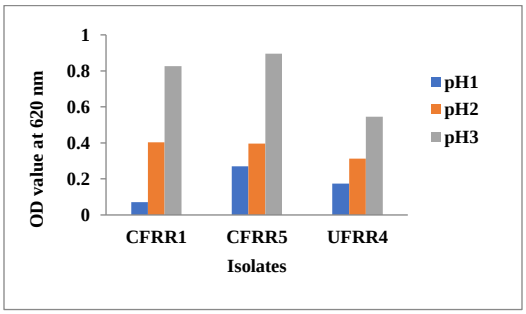

Fig. 3. Acid Tolerance of Isolates at Sixth Hour

Antibiotic susceptibility determines the sensitivity of the bacteria to specific antibiotics. Here, all tested isolates found sensitive to most of the antibiotics studied and showed intermediate towards Ciproflaxinonly. CFRR1 and UFRR4 were intermediate towards Imipenem and Erythromycin whereas UFRR4 intermediate towards Tetracyclin. Results indicate that isolates do not carry any transmissible antibiotic resistance genes, satisfying one of the key properties of probiotic bacteria.

Anti-microbial activity refers to the process of killing or inhibiting the pathogenic microorganisms. Only CFRR5 was active against *Listeria monocytogenes* was indicated by the clear zone of 1 mm positive inhibition as mentioned by Mulaw*et al.*,(2019) (Fig. 4).

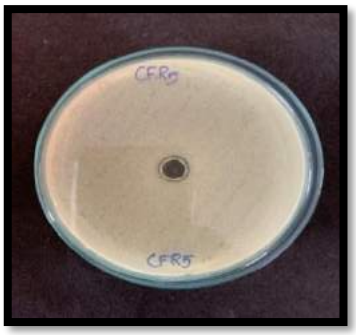

Fig. 4. Zone of Inhibition by CFRR5 against *Listeria monocytogenes*

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The isolate CFRR5 was sequenced and BLAST analysis indicated 99.39% of itssimilarity to *Bacillus albus.* The phylogenetic analysis of the identified isolate with its similar hits revealed the identified isolate is alsoclosely related to the *Bacillus albus, Bacillus wiedmanni*and *Bacillus cereus* (Fig.5).Submitted sequence to the GenBank of NCBI and obtained the identification as **MT186174**.

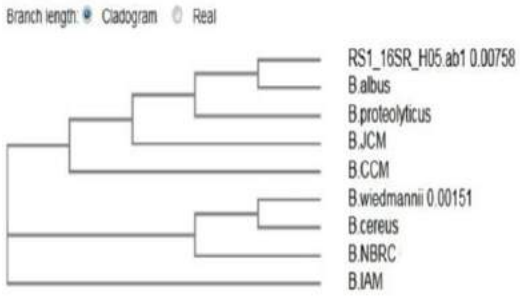

Fig 5: Phylogenetic analysis

The spore forming bacterial species from *Bacillus* genus are regarded as safe and used as probioticsas it can survive under different processing stress conditions. Unless a pseudo outbreak reported by Kniehl*et al.,* (2003) no case of foodborne infection caused by *B. cereus* when used as probiotics. Different*B. cereus* strains were studied for its beneficial effect on livestock including rabbit, piglets and sea cucumber (Trocino*et al.,* 2005; Altmeyer*et al.,* 2014; Li *et al.,* 2015).*In-vitro* inhibition of aqua pathogen(*Aeromonashydrophila*) was possible by the isolation of *B. cereus* strain JAQ04 as potential probiotic (Bernard *et al.,* 2013). The spore forming probiotic bacteria such as *Bacillussubtilis* and *Bacillus coagulans* helps to combat the infection caused by *Salmonella typhimurium* (Mazkour*et al.,* 2020). *Bacillus cereus, Bacillus clausii, Bacillus pumilus* in commercial probiotic products consisting of bacterial spores claimed the probiotic properties (Duc*et al.,* 2004). Also, the probiotic product of India is the ViBact (made up of genetically modified*Bacillus mesentericus*) acting as an alternate to B-complex capsules.

In the present study, the identified novel *Bacillus cereus* group strain (CFRR5) from cooked fermented red rice is classified as rod shaped Gram positive, spore forming, non-pathogenic bacteria which can survive in 0.3 % of bile salt concentration, tolerate pH 3 up to 6 h, withstand low and high temperature, lacks antibiotic resistance genes, active against the common foodborne pathogen. This catalase positive bacterium defensing against hydrogen peroxide also indicate that it can cross protect the establishment of catalase deficient species facing oxidative stress in the gastrointestinal tract (GIT).

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Conclusions

Among various species of probiotics, the spore forming bacteria have high demand in the global market as it can survive at ambient temperatures during desiccation without the loss of its viability, avoid the use of expensive technologies such as freeze drying, offers the possibility of spores mixing with the powder. Though many *Bacillus* speciesreportedas pathogenic but still there are unexplored spore forming non-pathogenicnovel strains which could serve as eitherfood or feed supplements but emphasizing its safety and quality is relevant in the view of public health.

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Article 5

Ace2 Mediated Inhibition of Sars-Cov-2: Insights From Molecular Interaction Investigations

Subhashini Ramakrishna, Cellciya J, Ashwitha G, Gowthamen V, Hemachandran G, VarunKrishnaa J, Meena S, Nivetha B, Sujitha M, Veeralakshmi S and Sumathy R.

Abstract

Currently, an entire world is facing the economic crisis because of the ongoing pandemic caused by the recently identified coronavirus, Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2). This virus spread fast from human to human instigated the scientific community infinding new vaccines.SARS-CoV-2entry into host cells is mediated by the angiotensin converting enzyme 2 (ACE2).The identified vaccines are currently used for the treatment but shortage of the vaccine and its unknown effects necessitates to find out the potential inhibitors from natural source using *in silico* approaches is time saving approach. In the present study, the efficiency of the reported compounds of lemon peel essential oil is analysed for its anti-viral activity against ACE2 receptor and also compared itsefficiency against drugs in usage. Molecular docking analysis revealed that Neral and Geranialshowed higherinteraction efficiency and further wet lab analysis of these compounds provide the natural prevention/cure.

Keywords Coronaviruses, SARS-CoV-2, ACE2, Spike glycoprotein, Essential oil, Molecular docking

1. Introduction

Coronaviruses (Co-V) are one of the largest genomes among RNA viruses belonging to the family *Coronaviridae which* are enveloped, non-segmented, made of single stranded positive sense RNA genomes comprising 25-32 kilobases in length. Amongst seven human coronaviruses, OC43, 229E, NL63 and HKU1 cause mildillness whereasSevere Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV) had significant public health concerns due to their zoonotic emergence, crossed the species barrier andcaused high pathogenicity with 9.6% and 34.3% fatality rate during 2003-2004 and 2012 respectively (WHO 2003; WHO 2020). SARS-CoV-2 was first identified and reported in Wuhan, China during 2019.The sourceor the intermediate from where this virus spread is still uncertain.The complete genome of SARS-CoV-2 revealed that it is most closely related with bat SARS (bat CoV RaTG13) and next closest with bat SARS-like coronaviruses such asbat-SL-CoVZC45 and bat-SL-CoVZXC21 (Yosra *et al.,* 2020).Based on its genetic relatedness, SARS-CoV-2linked to originate from bats (WHO 2020; Malik 2020). The viral genome encodes for both structural and non-structural protein. The four main structural proteins are spike (S) glycoprotein, small envelope

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(E) glycoprotein, membrane (M) glycoprotein, nucleocapsid (N) protein and also several accessory proteins (Jiang *et al.,* 2020). The N protein is a structural protein that binds to the coronavirus RNA genome creating a capsid around the enclosed nucleic acid, also involved in viral replication cycle and further cellular responses. Another important M protein is the most abundant structural protein that defines the shape of the viral envelope (Schoeman and Fielding, 2019). Its binding stabilizes N protein forming N protein-RNA complex inside the internal virion promoting the complete viral assembly. The E glycoprotein is the smallest of the all structural proteins playing its role in the production and maturation of the virus (Schoeman and Fielding, 2019; Tai *et al.,* 2020).

The S glycoprotein is a transmembrane protein found in the outer portion of the virus which forms homotrimers facilitating the attachmentand entry of the enveloped viruses to the host cell surface receptor namely angiotensin-converting enzyme 2 (ACE2). The virus not only affect lungs but also the other host organs where the receiver receptor is expressed such as inmyocardial cells, kidney proximal tubule cells, upper esophagus, bladder urothelial cells, stratified epithelial cells, and absorptive cells of ileum and colon (Xu*et al.,* 2020). SARS related coronaviruses interact directly with ACE2 through their SB (S1-binding domain B) to enter target cells. The SARS-CoV-2 SB engages human ACE2 (hACE2) with comparable affinity than SARS-CoV SB. The binding domain (331 to 524 residues) of S glycoprotein from SARS-CoV-2 attaches strongly with human ACE2and bat ACE2 (Tai *et al.,* 2020) followed by the fusion of the viral membrane with host cell (Walls *et al.,* 2020).

Most cases of SARS-CoV-2 are initiated by low virus loads, which cause asymptomatic to mild or moderate symptoms including fever, dry cough, body pain, headache, etc. Severe cases are associated with respiratory illness which may also lead to death. Available drugs are used for the treatmentsuch as anti-HIV, anti-malarial and anti-viral, etc. Aerosol inhalation of α-interferon was also used for the treatment at beginning but its effect last only for 14 days. However, combination of interferon with steroid drugs accelerated lung repair and increases oxygen survival levels. Studies also concluded that aerosol inhalation of interferon does not have any beneficial effect on patients (Teesa and Marjolein, 2019; Mona*et al*., 2003).Natural compounds have been reported to possess multiple biological activities. Essential oils are extracts of potentially beneficial plants and screened against several pathogenic viruses including Influenza and other respiratory viral infections. Here, the efficiency of the reported compounds of lemon peel essential oil is analysed for its anti-viral activity against ACE2. Also, its efficiency is compared with the existing drugs used for the treatment, and standard to find out the natural remedy.

2. Material and Methods

1.1. Target Preparation

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PDB (Protein Data Bank) is a key in area of structural biology and genomics. The atomic coordinates of the protein structure of human ACE2 (1R4L) in complex with inhibitors namely(S,S)-2-{1-carboxy-2-[3-(3,5-dichloro-benzyl)-3H-imidazol-4-YL]-ethylamino}-4-methyl-pentanoic acid and 2-acetamido-2-deoxy-beta-d-glucopyranose was retrieved from this database (Fig. 1).

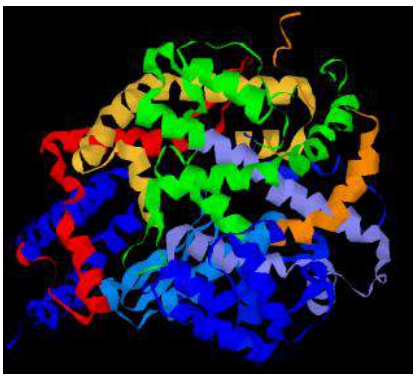

Fig. 1. 1R4L - Structure of ACE2

1.2. Ligand Preparation

ChemSketch, a molecular modeling program allowsthe chemical structures to display in two and three dimensions (2D and 3D) for understanding the chemical bonds and the nature of functional groups in addition to visualization (https://www.acdlabs.com). Chemical compound structures of lemon peel essential oilsuch as α-Pinene, Sabinene, β-Pinene, β-Myrcene,*p*-Cymene, Limonene, γ-Terpinene, Neral, Geranial, Hydroxychloroquine, Chloroquine (anti-malarial compounds), Lopinavir and Ritonavir (anti-HIV), Remdesivir (antiviral), Captopril (ACE2 inhibitor) and 1R4L ligands namely (S, S)-2-{3-(3,5-Dichloro-Benzyl)-3H-Imidazol-4-YL]-ethylamino}-4-Methyl-Pentanoic Acid, 2-acetamido-2-deoxy-beta-d-glycopyranose were obtained as .mol file format and 3D view (Fig. 2).

1.3. Structure File Format Conversion

Open Babel, an open-source chemical toolbox whichinter convert’s over 110 formats of chemical data (http://openbabel.org.in). The selected compounds were converted from .mol format to .pdb format using the file format conversion tool.

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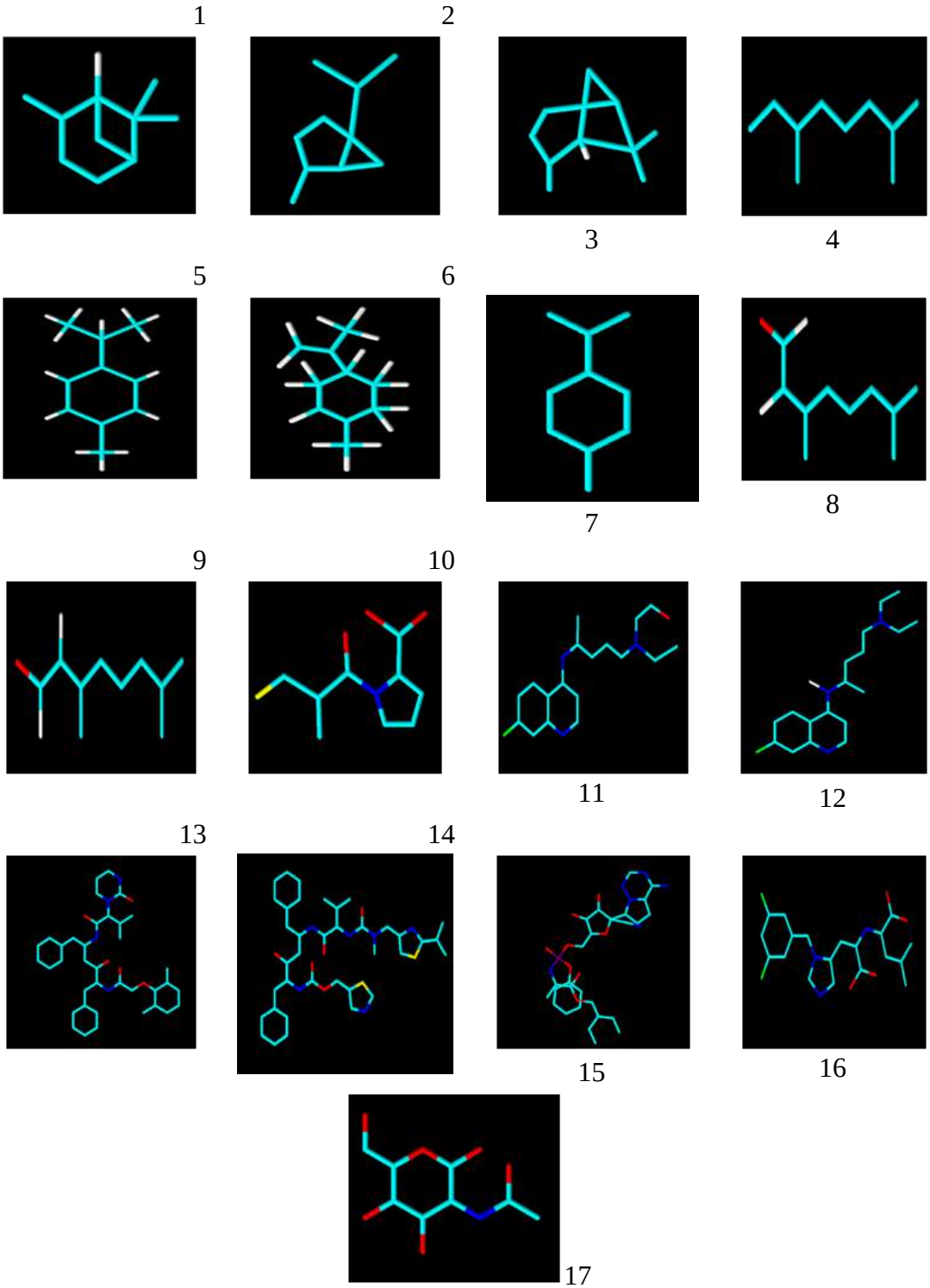

Fig. 2.Selected Compound Structures

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1.4. Active Site Analysis

CASTp server, an online resource used for locating the pockets present on the protein surfaces which is important for studying its features and functional regions. The active site residues of the selected targetwere predicted using this server.

Molecular Interactions

AutoDock, molecularmodeling simulation software is effective for protein-ligand docking andit is considered as one of the most citied docking software. The molecular interaction between the selectedtargetwitheach compound was analysed with this docking platform.

Molecular Visualization

Molegro Molecular Viewer is widely used for 3D visualization of macromolecule and small molecules. Interactions between moleculeswere viewed using this program.

ADME Properties

SwissADME predict models for physicochemical as well as pharmacokinetic properties (http://www.swissadme.ch). The input zone comprises a molecular sketcher which enables to import, draw and edit 2D chemical structures. The output panel displays the drug likeness properties of the selected chemical compounds.

2. Results and Discussion

2.1. *In silico* Inhibitory Effect of the Natural Compounds against ACE2

Targeting ACE2 is the host specific therapy to block the SARS-CoV-2 entry into host cells. The primary focus of this research is to identify potentialnatural therapeutics targeting the host receptor for the treatment of COVID-19.

Active site residues of the selected target (1R4L) predicted using theCASTp server was comparedagainst the functional amino acids of the protein reported in the UniProt database(https://www.uniprot.org). The identifiedresidues present in the functionally importantregion of the protein is bolded **(HIS345, PRO346, THR347, ALA348, ASP368, HIS374, GLU375, HIS378, ASP382, HIS401, GLU402, GLU406, THR449, LEU503, PHE504, HIS505, TYR510, PHE512, TYR515,**ARG518, THR519, GLN522).The docking analysisof the target with each compound was performed.The active site residues involved in the interaction is bolded and those residuesforming hydrogen bond interaction isbolded red colourwhereas non-active siteresidues involved is such interaction is given in red only (Table 1 and 2)

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Name of the Compound	Parameters				
	Binding Energy (kcal/mol)	**Cluster RMSD**	**Inhibitory constant (Ki)**	**H-bonds**	**Protein Ligand Interactions**
α-Pinene	-6.04	0.00	37.55 uM	-	TRP477, TRP478, LYS481, LEU456, ASP499, PRO500, ALA264, HE452, SER502, **LEU503**, TRP271, LY272, ALA264, ARG169, TRP165
Sabinene	-6.94	0.00	8.13	-	TRP477, PRO500,LYS481,TRP478, ASP499, ARG169,**LEU503**,TRP271
β- Pinene	-4.08	0.00	1.02 mM	-	**TYR515, HIS505, PHE504, HIS378, HIS374, GLU375,ALA348,THR347, PRO346, HIS345, PHE504**
β-Myrcene	-5.85	0.00	51.72 uM	-	TRP477, TRP478, LYS481,EU456, PRO500, ASP499, ALA501, PHE452,VAL487, ALA264, TRP165, MET270, TRP271, ARG169, **LEU503**, SER502
p-cymene	-4.64	0.00	400.27 uM	-	SER511, **TYR515**, ARG514,**GLU402, HIS378, GLU375, HIS505, TYR510, PHE504, HIS345, PRO346, THR347, ALA348**
Limonene	-4.71	0.00	353.06 uM	-	**HIS374, GLU375, HIS345, PRO346, THR347, ALA348, HIS378, GLU402, PHE504, HIS505, TYR510, ARG514, TYR515**
γ-terpinene	-4.60	0.00	422.44 uM	-	**PRO346, HIS345, PHE504, HIS505, TYR510,** SER511, ARG514,**TYR515,** THR347, **GLU375, ALA348, HIS378, GLU402**
Neral	-5.04	0.00	203.00 uM	3	ARG273, ARG518, **TYR515, HIS374,GLU402, HIS505, GLU375, HIS378, HIS345, PRO346, THR347, ALA348**
Geranial	-5.07	0.00	193.63 uM	4	ARG273, **HIS345, HIS505, TYR515, PRO346, THR347, ALA348, HIS374, GLU375, HIS378, GLU402**
Nafamostat	-10.72	0.00	13.97 uM	3	ARG518, **GLU406,** ARG514,**GLU402,TYR515**, SER409, **HIS505**, ARG273(2), PHE274,**HIS378, ALA348, HIS374, THR347, GLU375, HIS345, PRO346,** THR371, LEU370, ASP367
Hydroxychloroquine	-7.62	0.00	2.6 uM	3	THR362, MET360, CYS361,CYS344, **PRO346,** CYS344**, HIS345, ASP368, GLU375,** THR371, ASN149,**HIS505, TYR515,** ARG273
Chloroquine	-6.94	0.00	8.16 uM	-	**HIS378, HIS374, GLU375, ALA348, THR347, PRO346, HIS345, TYR515,** ARG273
Lopinavir	-11.99	0.00	1.63 uM	2	**HIS378, TYR510, GLU402, ARG514,GLU375, HIS374, HIS505, TYR515,** ARG518, **GLU406, HIS505,**SER409, ARG273, PHE274, THR445, TYR127, **LEU503,** TRP211, **THR515, HIS345 PHE504,** ASP368
Ritonavir	-	-	-	-	-
Remdesivir	-10.28	0.00	29.26 nM	3	**ALA348, GLU375, HIS378, HIS374,** THR371, **GLU406,** SER409, ARG518, LEU370, ASP367, **PRO346, TYR515, HIS345, TYR510, PHE504, HIS505,** ASP367, ARG273, PHE274, TRP271 ASN277, ASP269, GLY269
(S,S)-2-{1-Carboxy-2-[3-(3,5-Dichloro-Benzyl)-3H-Imidazol-4-YL]-Eethylamino}-4-Methyl-Pentanoic Acid	-9.54	0.00	102.07 uM	3	GLU206, ARG518,THR445,GLU402, **HIS374,** LEU370**, TYR515,** THR371, **HIS378,** ARG514, PHE274**,** ARG273, **HIS505, HIS345, TYR510, PHE504**
2-acetamido-2-deoxy-beta-D-glucopyranose	-6.75	0.00	11.21 uM	5	ARG518**, HIS374, HIS378, TYR515,** PHE274, ARG273**, HIS505, HIS345, PHE504**

Table 1: Docking Analysis of the Target with the Natural Compounds

α-Pineneinteraction with the target (1R4L) revealed that 15 residues TRP477, TRP478, LYS481, LEU456, ASP499, PRO500, ALA264, PHE452, SER502, LEU503, TRP271, GLY272, ALA264, ARG169, TRP165 were found interacting with the compound. On docking with sabinene, 8 residues namely TRP477, PRO500, LYS481, TRP478, ASP499, ARG169, LEU503, TRP271 were interacting with this compound. In both cases, only one active site residue namely LEU503 found interacting with compounds.

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Interaction of the target with β-Pinene revealed a total of 10 active site residues namely **HIS345,PRO346, THR347,ALA348, HIS374,GLU375,HIS378**, **PHE504, TYR515, HIS505** interacted with the compound at the binding energy value of -4.08 kcal/moland1.02 mMinhibitory constant. On docking with β-Myrcene showed 15 residues such as TRP477, TRP478, LYS481, LEU456, ASP499, ALA501, PHE452, VAL487, ALA264, TRP165, MET270, TRP271, ARG169, LEU503, SER502 interacted with the compound. Similar to α-Pinene and sabinene, only LEU503 is the active site residue interacted with β-Myrcene. The docking analysis of*p*-Cymene revealed that 15 residues (SER511, **TYR515**, ARG514, **GLU402, HIS378, GLU375, HIS505, TYR510, PHE504, HIS345, PRO346, THR347, HIS378, GLU375, and ALA348**) were found interacting. All residuesinvolved in the interaction are the active site residues except SER511 and ARG514.

Interaction between ACE2 and Limonene showed that 12 active site residues (**HIS345, PRO346, THR347, ALA348, HIS374, GLU375, HIS378, GLU402, PHE504, HIS505, TYR510, ARG514, TYR515**) involved in the interaction at binding energy value of -4.71 kcal/moland 353.06 uMinhibitory constant. The docking analysis of the target against γ-Terpinene showed that **PRO346, HIS345,PHE504, HIS505, TYR510,** SER511, ARG514, **TYR515**, THR347, **GLU375, ALA348, HIS378, GLU402** were interacting. Amongst, SER511, ARG514 and THR347 are non-active site residues whereas all the other residues involved in the interaction are the active site residues.

The docking analysis between the selected target and Neral showed that about 10 active site residues were (**ARG273, ARG518, TYR515, HIS374, GLU402, HIS505, GLU375, HIS378, HIS345, PRO346, THR347, ALA348**) found interacting. Three residues namely ARG272, ARG518, TYR515 formed hydrogen bond interaction, out of which only TYR 515 is the active site residue. The molecular interaction between the target and Geranial revealed that 10 residues namely ARG273, **HIS345, HIS505, TYR515, PRO346, THR347, ALA348, HIS374, GLU375, HIS378, GLU402** were found interacting. Out of which, 3 active site residues namely HIS345, HIS505, TYR515 and an non-active site residueARG273formed hydrogen bonding

The interaction analysis between the target and the standard indicated that 16 residues such as ARG518, **GLU406**, ARG514, **GLU402, TYR515**, SER409, **HIS505**, ARG273 (2), PHE274, **HIS378, ALA348, HIS374**, **THR347**, **GLU375, HIS345, PRO346**, THR371, LEU370, ASP367 were interacting. Amongst, only 11 residues are active site residues. HIS345 and ARG273 (2) alsoformed hydrogen bond interaction (the later formed hydrogen bond interaction twice). Molecular interaction between Hydroxychloroquine and the target showed that 16 residues were found as interacting residue. Amongst, only 6 residues are the active site residues such as **PRO346, HIS345, ASP368, GLU375, HIS505 and TYR515** whereas the residue at the position 345, 505 and at 273 also had hydrogen bond interaction with the compound.

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On docking with Remdesivir, 23 residues namely **ALA348, GLU375, HIS378, HIS374**, THR371, **GLU406**, SER409, ARG518, LEU370, ASP367, **PRO346, TYR515**, **HIS345, TYR510, PHE504, HIS505**, ASP367, ARG273, PHE274, TRP271, ASN277, ASP269, GLY269 were found interacting and only 11 residues are present in the active site of the protein. In addition, TYR515, HIS345 (active site residues)and ARG518 had hydrogen bond interaction with the compound. On dockingwith (S,S)-2-{1-Carboxy-2-[3-(3,5-Dichloro-Benzyl)-3H-Imidazol-4-YL]-ethylamino}-4-MethylPentanoic Acid, resulted with 16 interactions formed by residues namely GLU206, ARG518, THR445, GLU402, **HIS374**, LEU370, **TYR515**, THR371, **HIS378,** ARG514, PHE274, ARG273, **HIS505,HIS345, TYR510, PHE504**(only 8 are active site residues). TYR515, HIS378, HIS345 also had hydrogen bond with the receptor protein. Interaction studies between 2-acetamido-2-deoxy-beta-D-glucopyranose and the target revealed that 9 residues namely ARG518, **HIS374, HIS378, TYR515,** PHE274, ARG273, **HIS505, HIS345, PHE504** showed interactions. TYR515, HIS505, HIS345 and ARG518 also formed hydrogen bond interaction.

Molecular interaction analysis of the target with all compounds revealed thatα-Pinene and Sabinenehave shownhigher binding score than other compounds whereas only one active site residue LEU503 involved in the interaction. Similarly, β-Myrcene also showed its interaction with the same active site residue. Comparatively, β-Pinene, *p*-cymene, Limonene and γ-terpinene showed higher number of active site residues involved in the interaction whereas none of them formed hydrogen bond interaction.

Lemon peel essential oilcompounds namely Neral and Geranial showed more number of interactions and hydrogen bonding is given in Fig. 3 and 4.Although possessed lower binding energy score which means the stronger binding affinity but only less number of active site residues found interacting with Hydroxychloroquine, Chloroquine, Lopinavir,Remdesivir.

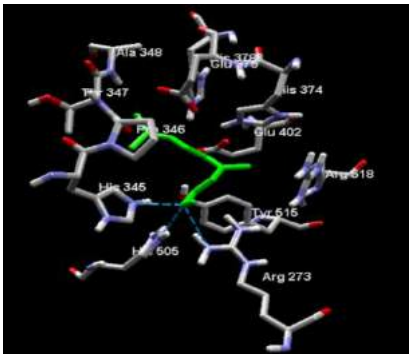

Fig. 3. Interaction between the target and Neral

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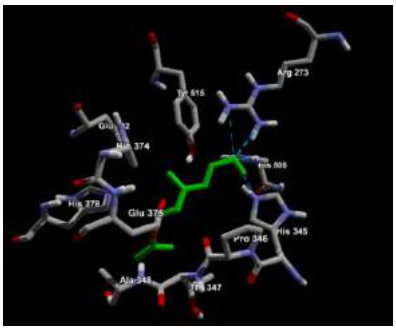

Fig.4. Interaction between the target and Geranial

Drug-likeness property of the selected compounds analysed by Swiss ADME server showed that Lopinavir, Ritonavir, Remdesivir with molecular weight greater than 500 g/mol. In addition, Ritonavir found non-interacting with the target. Also, Remdesivir formed interactions with H-bond acceptors more than 10.All natural compounds showed good water solubility equivalent to the control and better than anti-HIV, anti-malarial and anti-viral drugs. According to Lipinski's rule for drug likeness, any molecule having molecular weight greater than 500 g/mol, hydrogen bond accepting atoms > 5, hydrogen bond donating atoms > 10, logP> 5 is not considered as good pharmaceutical agent with oral activity. No violation of the rule by compound indicates its pharmacokinetic nature. In the present study,Lopinavir, Ritonavir, Remdesivirviolated the rule (Table 2).

Existing therapies have been approved for treating corona viral infections but disadvantage is that these therapies are broad spectrum drugs which cannot kill the virus in a targeted manner and one should never underestimate its side effects. The nucleoside analogue Remdesivir has not yet been approved for marketing in any country (Canrong*et al*., 2020). Hydroxychloroquine increases the risk of serious cardiac toxicity, cardiac dysfunction whereas Lopinavir and Ritonavir including Hydroxychloroquine increases the risk of diarrhoea, nausea and/or vomiting(Ariel *et al*., 2020). Prolonged or high level dosage of the other anti-malarial drug can cause retinopathy, cardiomyopathy, myopathy, and neuropathy (Cássio*et al*., 2015).

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S.No	Name of the Ligand	Molecular Formula	Molecular Weight (g/mol)	H-bond Acceptors	H-bond Donor	LogP Value
1	α -Pinene	$C_{10}H_{16}$	136.23	0	0	2.63
2	Sabinene	$C_{10}H_{16}$	136.23	0	0	2.65
3	β- Pinene	$C_{10}H_{16}$	136.23	0	0	2.59
4	β-Myrcene	$C_{10}H_{16}$	136.23	0	0	2.89
5	p-Cymene	$C_{10}H_{14}$	134.22	0	0	2.51
6	Limonene	$C_{10}H_{16}$	136.23	0	0	2.72
7	γ-Terpinene	$C_{10}H_{16}$	136.23	0	0	2.73
8	Neral	$C_{10}H_{16}O$	152.23	1	0	2.47
9	Geranial	$C_{10}H_{16}O$	152.23	1	0	2.51
STANDARD						
10	Nafamostat	$C_{19}H_{17}N_5O_2$	347.37	4	4	1.35
ANTI-MALARIAL						
11	Hydroxychloroquine	$C_{18}H_{26}ClN_3O$	335.87	3	2	3.58
12	Chloroquine	$C_{18}H_{26}ClN_3$	319.87	2	1	3.95
ANTI-HIV						
13	Lopinavir	$C_{37}H_{48}N_4O_5$	628.80	5	4	4.22
14	Ritonavir	$C_{37}H_{48}N_6O_5S_2$	720.94	7	4	4.38
ANTI-VIRAL						
15	Remdesivir	$C_{27}H_{35}N_6O_8P$	602.58	12	4	4.81
1R4L INHIBITORS						
16	(S,S)-2-{1-Carboxy-2-[3-(3,5-Dichloro-Benzyl)-3H-Imidazol-4-YL]-Eethylamino}-4-Methyl-Pentanoic Acid	$C_{19}H_{23}Cl_2N_3O_4$	428.31	6	3	2.62
17	**2-acetamido-2-deoxy-beta-D-glucopyranose**	**$C_8H_{15}NO_6$**	**221.21**	**6**	**5**	**0.20**

Table 2: Lipinski's Rule of Five Properties for the Selected Compounds

Conclusion

Based on results, Neral and Geranial with its low molecular weight, no violation of Lipinski rule of five, possessing equivalent interaction efficiency as the standard drug and bound inhibitors facilitates further wet lab analysis which might be useful as a natural therapeutic agent for restricting the viral attachment via its cellular receptor.

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Article 6

Molecular Documentation of Fermentative *Saccharomyces* and Non-*Saccharomyces* Yeastfrom *Cocos Nucifera*: Assessing its Probiotic Properties

Subhashini Ramakrishnan, SrieeViswarubhiny A. Srivatsan S.M, Yuvashree U, Sruthila S, Aashik Ahmed M.G, Suganthi R and Nivetha B.

Abstract

Screening of multiple strains using *in vitro* assays is the most preferable choice for many researchers due to its simplicity and low cost approaches. Evenits intrinsic properties are appropriately validated by such assays. Many strains classified using molecular data upturn further workdue to their speed andspecificity. Probiotics are sustainable organisms able to survive in different compartments of gastrointestinal tract claiming its health benefits. Evaluation of the probiotic properties of strains using laboratory tests uplifts the usage of such cultures in developing functional products beneficial for the society. In the present study, *Torulaspora delbrueckii* and *Saccharomyces cerevisiae*screened from sap and toddy of *Cocos nucifera*was identified using molecular analysis and deposited it in GenBank(**KU949000** and **KU949001)**. Fermentative capacity of organisms and tolerance assays such as NaCl, temperature, acid,bile salt indicated its probioticcharacteristics. Identified conventional and non-conventional yeast as indigenous microflora is useful either as pure or mixed culture in theindustrial fermentation processes.

Keywords *Cocos nucifera*, Sap, Toddy, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, Fermentation

1. Introduction

Natural fermented beverages produced from either fruit juice or plant sap are popular alcoholic beverages. *Cocosnucifera*(coconut tree), amongst members of the family palmrae is widely distributedin peninsular India andoffers wide range of products including sap and toddy.Incising the inflorescence of the coconut tree oozes sap, this freshly tapped liquid tends to be very sweet and non-alcoholic until it ferment into toddy. In many societies, thetraditional fermented drinkis consumed predominantly before breaking the fast for health benefitsas it enhances the growth of beneficial organisms, nutritional properties, and also serves as sedative.Fresh coconut sap contains minimal percentage of sucrose and trace amount of other sugars serves as a rich substrate for the growth of the natural microbiota which in turn converts sap in to toddy, the fermented sap.

Earlier studies showed that different organisms from sap and toddy was confirmed based on its biochemical characteristics, evaluatedits acid production, withstanding capacity of organisms in acidic pH and bile salt, alcoholicresistance yielding higher concentration of

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ethanol, antimicrobial properties(Kadereand Kutima, 2008; Ziadi*et al.,* 2011;Satheese*t al.,* 2011). Lactic acidbacteria isolated from fermented toddy of *C. nucifera*with probiotic propertieswas reported as responsible for thepotential inhibition of food borne and otherhuman pathogens (Krishnamoorthy and Arjun 2012; Krishna Moorthy*et al.,* 2015). Probiotics consumed in the form of dietary supplements and/or foods is beneficialfor residents. Different yeastsaccomplish itsroleas essential in fermenting process but only a limited number of strains have been identified. Classifyingsuch eukaryotic probiotics from natural sources playing important role in fermentation improvises the quality of fermented products. Remarkably, the effort of understanding its presence in natural products multiplies its usage for health benefits and recognising its role extends the production values in industries. Current investigation means on finding the friendly microflora associated with the sap and toddy of *C. nucifera* and assessed its effectiveness as probiotic.

2. Materials and Methods

2.1. Isolation and Identification of BeneficialOrganisms

Fresh sap and toddy of *C. nucifera*collected from Coimbatore district, TamilNadu, India.Samples were collected in pre-autoclaved plastic container and procured to the laboratory in an icebox within 2 h and immediately stored at 4 ℃ to avoid fermentation.Known volume of 8.9% saline was prepared and autoclaved at 121℃ for 15 min. 1mL of each sample was homogenized in 99mL of pre-autoclaved saline.Transferred 1 mL of quot to each 9 mL of saline andserially diluted upto 10^{-7}.Then, 1 mL of appropriate dilutions were pipetted out on to the Yeast Peptone Dextrose Agar (YPDA) and Nutrient Agar (NA) followed by incubation for 24 h. Out of several colonies grown on different plates, eightcolonieswereselected randomly,quadrant streaked, subcultured repeatedly until thepure culturewas obtained and glycerol stock of all isolateswere maintained at -20℃.Gram reaction property and biochemical characterisations such as motility, catalase,hydrogen sulphide and indole production were examined.

2.2. Probiotic Assays

Obtained pure isolates from *C. nucifera* were subjected to different *in-vitro* assays to examine its efficacy as probiotic.

2.3. Carbohydrate Fermentation

Fermentative media was prepared and added with 0.1 g of sugar substrate (galactose, fructose, lactose, dextrose, sucrose) in each tube containing 10 mL of medium. Also, placed Durham tubes into the media to trap the released carbondioxide and sterilized for 15 min at 121°C. Tubes were then inoculated with the each isolate under study and incubated it for 24 h.

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2.4. NaCl Tolerance

Each isolate was inoculated separately in the broth containing different concentrations of NaCl (2%, 4% and 6.5%) and incubated for 24 h.

2.5. Temperature Tolerance

All isolates were allowed to grow under different temperature (15 °C, 30 °C and 42 °C) and measured its growth rate at 600 nmand recorded it Optical density (OD) valueafter 24 h of incubation.

2.6. Acid Tolerance

Freshly prepared 10mL of the respective broth at different pHconditions (1.5, 2, 3, 4, 5, 6) was maintained with 1N hydrochloric acid (HCL)and inoculated with 0.1 ml of the culture followedby incubation. The growth was measuredat two different time intervalsand recorded its OD values at 620 nm.

2.7. Bile Salt Tolerance

Freshly prepared Yeast Peptone Dextrose Broth (YPDB)with different concentrations of bile salts (0.5%, 1%, 2%) and inoculated with the culture suspensionfollowed by incubation. The growth was measured up to 6 h and recordedits OD value at 620nm respectively.

2.8. Anti-microbial Activity

Antimicrobial activity was determined by disc diffusion method. Agar plates were prepared and swabbed with each food-borne pathogens such as *Salmonella* spp., *Staphylococcus* spp., and *Escherichia* spp.. Sterile blotting paper discs dipped in the overnight culture broth was then placed onto the solidified agar plates, allowed for diffusion and incubated. Zone of clearance was observed after 24 h onwards.

2.9. Molecular Identification

Sequencedthe identified isolates where the trained personnel's used 18srRNA marker as an organism barcode for detecting the amplified DNA with DNA sequencing program. The obtained sequences werethen submitted to the blastnprogram and identified based on its similarity score and expected threshold value. The identifiedmolecular sequences were then depositedin the public repository of nucleotide sequences.

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3. Results and Discussion

3.1. Isolation, Morphological and Biochemical Characterisation of Isolates

Prokaryotic probiotics have been studied extensively whereas research on eukaryotic probiotics is limited. The research work engaged on identifying the eukaryotic beneficial organisms from *C. nucifera* with potential properties enhances valuable applications.The colour of the collected fresh sap was partially colourless while toddy was turbid (Fig. 1).Eight colonies (each four from sap (S1 to S4) and toddy (T1-T4)) wereselected based on its morphology (i.e., colour, size, margin and shape) and all are Gram (+)ve isolates.

Fig. 1.The Collected Sap and Toddy after Tapping

Biochemical properties ascertained for each isolate indicated that S3, S4, T2 and T4 isolates are only non-motile. Isolating eukaryotic organisms necessitated the confirmation of presence or absence of the essential catalase involves in the decomposition of intracellular hydrogen peroxide. Isolates S3, S4, T2 and T4 only produced effervescence. All isolates showed the absence of hydrogen sulfideand indole production except T3.

3.1. Probiotic Characterisation

Fermentative capability of isolates using different sugars as the substrate was verified by the production of acid and gas. Isolates such as S2, S3 and T2 fermented lactose whereas S3 produced gas. Maltose was fermented by all isolates but only S3 produced gas. Similarly dextrose was also fermented by all isolates and produced gases. All isolates fermented fructose andgalactose (except S1, S2 and T1) whereas S4 and T4 only produced gas during fermentation. Obtained result indicated that all isolates were able to survive at all concentrations of NaCl.Only S4 and T4 isolates exhibited maximum growth at 15°Cwhereas the growth of the other isolates at this temperature was found low and S1 was totally sensitive. All isolates grown at 30°C when compared to S4, T1 and T4 and the growth rate of S2, S4, T2, T3 and T4 was found low at 42 °C (Fig. 2).

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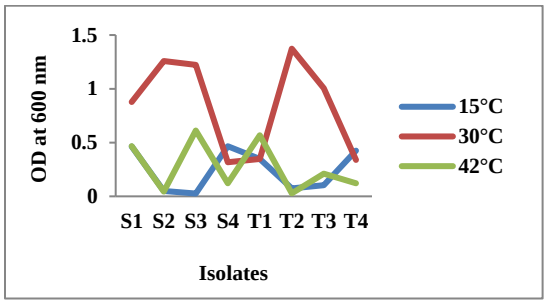

Fig. 2. Temperature Tolerance of Isolates

Secretion of hydrochloric acid in the stomach is the main barrier that affects the microbial growth. The pH of the stomach is anywhere between 1-2 and it can also rise up to 4-5 after the food consumption. Another prerequisite for the probiotic strainsafter its passage through the acidic conditions in the stomachis that must be able to tolerate the other sequential stress like bile salt concentration. Isolates (S4 and T4) endured its growth at their low temperature were analysed for its acid and bile salt tolerance. Both isolates tolerated low acidic and bile salt conditions but withstanding capacity of T4 isolate from toddywas high comparatively (Fig. 3 and 4).

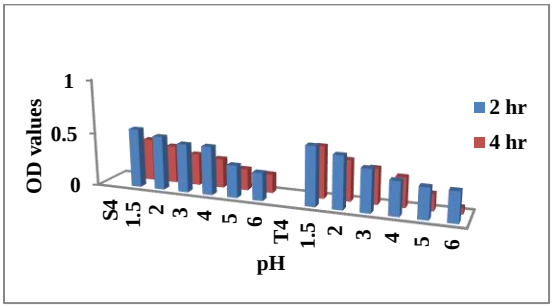

Fig. 3. Acid Tolerance of S4 and T4 Isolates

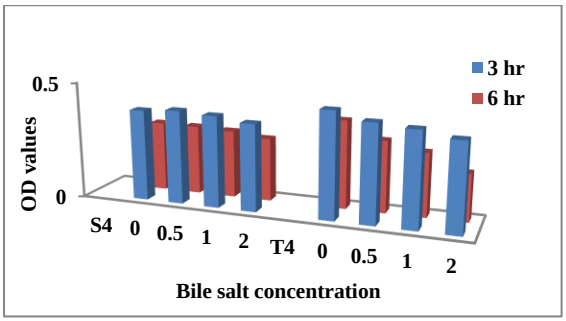

Fig. 4. Bile Salt Tolerance of S4 and T4 Isolates

3.2. Antagonist Property

All isolates examined for its antimicrobial activity against the common food borne pathogens infecting the gastro intestinal tractindicated absence of zonedue to its lacking in the production of bacteriocin or antibacterial compounds active against these enteropathogens.

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3.3. Molecular Identification of S4 and T4 Isolates

Different useful organisms are isolated from different sources, however molecular techniques is appropriate to identify it. S4 and T4 isolates (Fig. 5) weremolecular sequencedandBLAST analysisof the obtained sequence indicated organisms as*Torulaspora delbrueckii* (S4) and *Saccharomyces cerevisiae* (T4) based on its e-value and the similarity score. Sequences were then submitted to GenBank and obtained the identification as **KU949000**(S4 isolate from sap, 609bp)and **KU949001**(T4 isolate from sap, 584 bp).

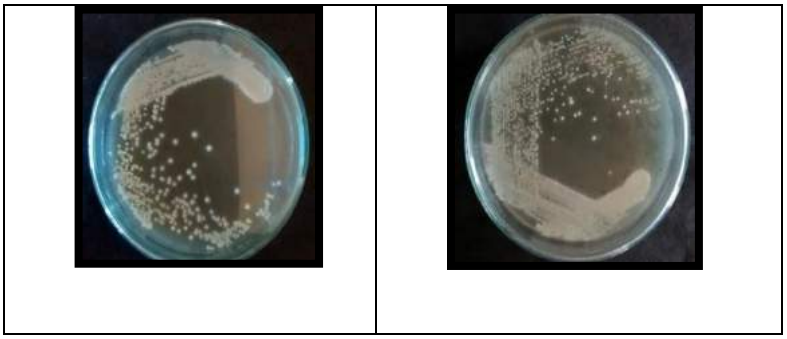

Fig. 5. S4 and T4 from Sap and Toddy of *C. nucifera*

Yeasts being important in fermenting foods and beverages, also confirmed for its numerous beneficial effects on health including probiotic activity, prevention and treatment of diseases, folate biofortification, bioavailability of minerals, detoxification of mycotoxins, food enrichment, lowering of cholesterol, antioxidative, antitumor properties, etc (Saloomeh*et al.*, 2010). *Torulaspora delbrueckii (S4) and Saccharomyces cerevisiae* (T4)from sap and toddy of *C. nucifera* are non-motile; degrade hydrogen peroxide, showed the absence of hydrogen sulfide production and indole accumulation. Both isolates utilized sugars as substrate indicates fermentative, able to withstand minimum to maximum concentrations of NaCl (2 % to 6.5 %).The starter culture strains catalyzing the conversion of sugars into alcohol and not produces off-flavors are the other significant factors during the production of fermentative products. The volatile sulphur containing compounds like hydrogen sulfide (H_2S) formed during fermentation is been associated with off-flavors and the higher concentration of indole produces plastic-like flavors in fermented products (Maria *et al.*, 2010).

Temperature is the main factor influencing the fermentative product quality. High temperature might favour the yeast growth but it decreases the productivity of the fermented product. S4 and T4 isolates survived at lower temperature might enhances higher concentration of esters which helps in maintaining the stability of flavor and aroma compounds during fermentation, and also better control over the microbial growth(Molina *et al.*, 2007; Beltran *et al.*,, 2006; Jackson 2014).

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The identified organisms were able to withstand the low acidic conditions signifying it as acid resistant strains having the capacity to produce organic acids and less sensitive to microbial spoilage(Costal *et al.,* 2015).Even the wine fermented with native organisms had its lowest acidity was reported by Justyna*et al.,*(2019). The physiological concentration of the bile salt ranges from 0.3 to 2% in the human intestine during digestion process. Although, the bile salt concentrations ranging between 0.15 and 0.5% is been recommended for probiotics(John 2000). The identified isolates possess its capacity to survive in the presence of bile salt up to 2 %. Bile Salt Hydrolase (BSH) is an enzyme that hydrolyses conjugated bile salts thereby reduces toxicity. Increased BSH activity by probiotic strains helps in reducing the cholesterol and decreases the body weight (Patrice and Matthias 2015).

Anywhere for large-scale fermentation, only commercial yeast strainsare usually used which is prepared by either *in house* or supplied by yeast producing companies.Spontaneous fermentation relies on indigenous microflora present in the raw material produces high quality and unique-flavored fermented products (Justyna*et al.,* 2019). Characterizing such efficient eukaryotic strains and preparing inactive dry yeast with stress tolerance are gaining popularity. *Torulaspora delbrueckii* possess good fermentation when compared to the other non-Saccharomyces yeasts andclaimed for optimizing wine parameters with respect to the usual Saccharomyces yeast (Laura et al., 2020).

Identified organisms from *C. nucifera* might be useful for industrial fermentation where it fermented all the sugars except lactose clearly indicate these organisms avoided mololactic fermentation as this process produces undesirable compounds and unsafe for lactose intolerants. Maintaining lower temperature during fermentation will also inhibit mololactic fermentation. However, further analyses are required on these obtained organisms from the specified sourcewith respect to the oenological parameters for industrial fermentative product making.

Conclusion

Identification of probiotics from alternative sources increases the possibility of identifying new strains. Various essential organisms in fermentation process are reported from different sources. Eukaryotic microorganismsaremainly used in large scale fermentation and identifying such probiotics from natural sources possesses wide applications which may enhance both the human wellbeing as well as the production values. The presence of *Saccharomyces*and non- *Saccharomyces*yeastin sap and toddy of *C. nucifera* confirms its fundamental importance in the fermentation process.However, further studies are requiredwith critical parameters of industrial processing which enhance the development of functional productsusing these organisms.

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Article 7

Phytochemical Analysis and Antioxidant activity of few *Ipomoea spp.,*

Gaayathiri Devi.E, Nisha.M. K and Selvapriya.K

Abstract

Ipomoea cairica L, *I. alba* L., *I. biloba*, *I.indica*(Burm) Merr and *I. hederifolia*L. which belongs to the family Convolvulaceae comprises approximately 1,600-1,700 species grouped in 55-60 genera were taken for this phytochemical study. The plants of the genus *Ipomoea* are reported as medicinally useful for treating different diseases and pathological conditions, in kidney ailments, constipation, colic and digestive disorders. Some species showed antimicrobial, analgesic, spasmolitic, spasmogenic, hypotensive, psychotomimetic and anticancer activities. The quantitative analysis of phytochemicals (protein, carbohydrate, total flavonoid and total phenol) and non-enzymatic antioxidants (total ascorbic acid, total tannin, total alkaloids and total polyphenol) were studied in the methanol extracts of the leaf and stem extracts of all the five species of *Ipomoea.* The quantitative phytochemical analysis and non-enzymatic antioxidants showed a significant amount of protein (9.27±0.04) in the *I. biloba* leaf, carbohydrate (16.3±0.59) and flavonoid (2.92±0.03) in the *I.indica*(Burm) Merr. Leaf, total phenol (6.55±55.4) in the *I. alba* L. stem and total ascorbic acid (9.50±14.9) in *I.indica*(Burm) Merr stem, total tannin (8.88±22.3) in the *I. alba* L. stem, total alkaloids (3.78±1.72) in the *I.indica*(Burm) Merr. Leaf and total polyphenol (9.58±2.2) in *I.biloba* leaf in the methanolic extract.

Keywords Antioxidants, Convolvulaceae, *Ipomoea*, Methanolic extract, Quantitative analysis.

1. Introduction

Phytochemicals are the natural bioactive substance obtained from the plants along with its nutrients and fibres to form an integrated part of defense system against various disease and stress condition (Koche., 2010). Phytochemical screening involves the evaluation of various chemical components in plants. These chemical components can interchange in quality and quantity in various plants. There are two types of metabolites produced in plants called as primary metabolites and secondary metabolites which are important for the plants regular metabolism such as growth and development. Phytochemicals present in the different plant parts like bark, leaves, stem, root, flower, fruits, seeds, etc., are used up by the local peoples for healing of certain disorders (Ugochukwu *et al.*, 2013). Convolvulaceae, the morning glory family of flowering plants is the major group of Angiosperm which includes 57 genera and about 1,600 species widespread in both tropical and temperate areas. *Ipomoea*, is the largest genus in the flowering plant of the family Convolvulaceae which contributes about 600-700 species all over the world. The genus is rich in many potential bioactive

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compounds such as alkaloids, lignins, glycolipids and phenolic compounds.The purgative property of this genus is due to glycoresins, an important chemo-taxonomical marker present in this family (Rogelio, 1995). The plants of the genus *Ipomoea* have long been used in folk medicine for the treatment of a wide variety of pathological conditions, showing antiinflammatory, antimicrobial, anticancer activities, analgesic processes, spasmolitic, spasmogenic, hypotensive, psychotomimetic and also includes they used to treat kidney ailments, constipation, colic and digestive disorders. Due to lack of knowledge about the utility of the plant, these species are rapidly destroyed from various places. Hence,The present investigation was carried out to investigate the quantitative and Non- enzymatic antioxidant activities of themethanolic extracts of leaf and stem of *Ipomoea indica, I. alba ,I.cairica, I. hederifolia and I.biloba.*

2. Materials and Methods

2.1. Collection and Preparation of plant material

The fresh and healthy *Ipomoea*species (*Ipomoea indica, I. alba, I.cairica and I. hederifolia*) leaves and stem were collected from Gudalur, Nilgiris, Tamil Nadu, India and *I.biloba*, was collected from Puthuvype beach, Kochi, Kerala during winter season (December, 2019). The plant materials were washed and dried in shade for 7 days and ground into a powder form using electric blender. The powdered samples were stored in glassware container until the time of extraction.

2.2. Extraction of plant sample

The powdered samples (10g each) were weighed and soaked in 100ml methanol in a conical flask stopped with rubber cork and left undisturbed for 24 hours. It was then filtered off using sterile filter paper (Whatman No.1) into a sterile conical flask.

2.3. Quantitative estimation of phytoconstituent

The biochemical parameters like Carbohydrates, Protein, Total phenol and Flavonoid were quantified in the leaf and stem samples of five *Ipomoea* species.

2.4. Estimation of Carbohydrate

Quantitative estimation of carbohydrate was carried out using the method of Hedge and Hofreiter, 1962. Anthrone reagent was prepared by dissolving 200 mg anthrone in 10 ml ice cold 95 per cent sulphuric acid was dissolved to prepare fresh anthrone reagent. Four ml of anthrone reagent was added and the reaction mixture was heated for 8 mins and cooled rapidly. The absorbance of the green coloured solution was measured at 630 nm using spectrophotometer using D-glucose as standard.

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2.5. Estimation of Protein

To 0.1 ml of sample add 5 ml of alkaline copper reagent and shaken well. Then the mixture is allowed to stand for 10 mins. Then 0.5 ml of Folin–ciocalteau reagent were added, shaken and incubated at room temperature in the dark for 30mins. BSA was used as standard. The blue color developed was read at 620 nm in a spectrophotometer (Lowry *et al.,* 1951.)

2.6. Estimation of Total Phenols

Phenolic compounds were estimated using the method of Mallick and Singh, 1980. To 1.0 ml of the extract, 1.5 ml of Folin- Ciocalteau reagent was added and placed in a room temperature for exactly five minutes. The tubes were diluted with 4 ml of distilled water and 2ml of 20% sodium carbonateand made up to 25 ml. The tubes with solutions were warmed for 1 minute then cooled. A blue colour was developed in each tube and absorbance was measured at 760 nm using concentrations of catechol as standard.

2.7. Determination of Total Flavonoid content(Shi *et al.*,2012).

To 1 ml of the extracts, 4 ml of water is added to 0.4ml of $NaNO_2$ and 0.3ml of $AlCl_3$. The mixture was then incubated at room temperature for 5 min. 2 ml of sodium hydroxide (1M) was added and the final volume of the mixture was brought to 10 ml by adding distilled water. The absorbance of sample and blank were determined at 510 nm by UV-VIS Spectrometer. The total flavonoid content was expressed in terms of mg rutin equivalents (RE) / g of sample.

2.8. Non - enzymatic antioxidant activity

Total Ascorbic acid, Total tannin, Total alkaloid and Total polyphenol were the non-enzymatic antioxidants analysed in leaf and stem of *Ipomoea* species.

2.9. Estimation of Total Ascorbic Acid (Roe and Keuther, 1943)

To 0.1 ml of extract 4% TCA was added and the volume was made upto 2.0 ml. The suspension was thoroughly shaken and filtered, followed by the addition of 0.5 ml of DNPH reagent and incubated in water at 37°C for 3 hoursresulting in the formation of osazone crystals and then cooled. The crystals were dissolved in 2.5ml of 85% sulphuric acid and the tubes were shaken thoroughly, cooled in ice and left for 30 mins for the colour formation. Using a spectrophotometer the absorbance was measured at 540nm.The concentration of ascorbate in the samples were calculated and expressed in terms of mg/g of sample.

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2.10. Estimation of Total Tannin (Folin and Ciocaltea Method, 1927)

The tannins were determined by Folin and Ciocalteau method. 0.1ml of the extract was added with 7.5ml of distilled water, 0.5ml of Folin – Ciocalteau reagent, 1ml of 35% sodium carbonate solution and diluted to 10ml with distilled water. After shaking the mixture well, it was kept for 30min in the room temperature.Absorbance was measured at 725nm and the distilled water was used as a blank. The results of tannin are expressed in terms of tannic acid in mg/g of extract.

2.11. Estimation of Total Alkaloids (Bromocresol green method)

To 0.1 ml of the extract, 5 ml of phosphate buffer (pH 4.7) and 5 ml of Bromocresol green solution (BCG) were added.The mixture was shaken with 4 ml of chloroform. The absorbance was measured at a spectrum of 470 nm against the blank prepared as above but without Atropine. Concentration was calculated using atropine as standard and expressed as mg of AE/g of extract sizes.

2.12. Estimation of Total Polyphenol (Malick and Singh, 1980)

The extracts with different concentrations were taken and the total volume made to 3 ml with distilled water. Then add 0.5 ml of Folin ciocalteau reagent and 2.0 ml of sodium carbonate A blue colour develops and warmed for 1 minute, cooled and the absorbance was measured at 650 nm against a reagent blank. Concentration was calculated using catechol as standard and expressed as μg of catechol equivalents/ g of sample.

2.13. Statistical analysis

All the analyses were conducted in triplicates. The Quantitative and antioxidant values for the leaf and stem extracts were evaluated with the one-way ANOVA and Tukey's Multiple Range Test .P values less than 0.05 were considered to be statistically significant. Values were expressed in means ± SD.

3. Result and Discussion

The quantitative and antioxidant analysis of five *Ipomoea* species revealed the presence of most of the tested major phytoconstituents. Table.1 shows the results of the quantification of estimated phytoconstituents.

3.1. Quantitative Analysis

Carbohydrate

The carbohydrate content of the crude extracts was determined with reference to the D-glucose stock standard. The significant amount of carbohydrate was determined in the leaf of *I. indica* (16.3±0.59), followed by the stem of *I. hederifolia* (15.4±0.7) and *I. alba*

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(15.3±1.5). The amount of carbohydrate present in the samples was expressed in (mg/g). Essiett and Ukpong 2014 reported the carbohydrate contents of *I. involucrate* (71.9%), *I. batatas* (73.7%) and *I. triloba* (77.42%) is relative high. The present result is in accordance with Uddin *et al.,* 2017 who reported the maximum amount of total carbohydrate was found to contain in *I.carnea* (17.71 ± 0.01 g% dwt) followed by *I. aquatica* and *I. batatus* (11.12 ±0.04 g% dwt and 7.57 ± 0.01 g% dwt), respectively.

Protein

Proteins were found to be higher in all the five leaf extracts when compared to the stem extracts. Proteins were observed in higher quantity in the leaf extracts of *I.biloba*(9.27±0.04) and *I. cairica* (9.14±0.23) compared to all other extracts. The result of the protein was expressed as (mg/g). BSA serves as a standard. The highest amount of total protein (27.25 ±0.02 g% dwt) was found in *I. batatus* and least amount of total protein (25.35 ±0.02 g% dwt) in *I. aquatica* plant. While in *I. carnea* the quantity was moderate (25.92± 0.01 g%dwt). Mangesh, 2015 reported the presence of protein content of 2.25 gm in *Ipomoea alba* L.

Phenol

High quantity of phenol was detected in the stem extracts of *I. alba* (6.55±5.54 GAE/g) followed by *I.cairica*(5.84±12.6 GAE/g) when compared to all other extracts. Similarly, Gopukumar (2013) reported the total phenol content was 920 µg / dl in *I. biloba.* According to Uddin *et al*., 2017 *I. batatus* contains the highest amount of phenolics (20.25 ± 1.13mg g-1dwt), *I. carnea* contains the least amount of phenolics (15.33 ± 0.17 mg g-1dwt) and *Ipomoea aquatic* contains moderate amount of phenolics (16.83 ±0.87mg g-1dwt).

***Ipomoea* species extracts**	**Carbohydrate (mg/g)**		**Protein (mg/g)**	**Phenol (RE/g)**	**Flavonoid (GAE/g)**
I.cairica(L)	Leaf	14.7±0.95	9.14±0.23	1.65±30.9	2.91±0.09
	Stem	13.7±3.1	7.53±0.26	5.84±12.6	2.57±0.08
I.alba(L)	Leaf	12.0±0.56	8.76±0.24	1.68±86.1	2.85±0.04
	Stem	15.3±1.5	7.02±0.59	6.55±55.4	2.71±0.07
I.biloba(L)	Leaf	14.9±2.13	9.27±0.04	1.13±10.5	2.88±0.02
	Stem	10.0±0.8	8.44±0.40	1.76±38.3	2.46±0.04
*I.indica*Burm (Merr)	Leaf	16.3±0.59	7.89±1.06	3.79±66.3	2.92±0.03
	Stem	13.9±0.6	5.39±0.33	3.21±6.11	2.77±0.06
I.hederifolia(L)	Leaf	11.8±2.38	6.53±0.05	4.43±8.18	2.90±0.08
	Stem	15.4±0.7	4.51±0.54	3.52±68.8	2.51±0.09
SED CD(P<0.05)		1.25 2.96	0.39 0.13	67.9 151.3	0.058 1.41

Table. 1 Quantitative analysis of phytochemical constituents of methanol extracts of leaves and stems of *Ipomoea* species

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Flavonoid

The flavonoid content was found to be high in all the leaf extracts of *I. indica* (2.92±0.03 RE/g), *I.cairica* (2.91±0.09 RE/g), *I. hederifolia* (2.90±0.08 RE/g), *I. biloba* (2.88±0.02 RE/g), *I.alba* (2.85±0.04 RE/g) when compared to the stem extracts. Uddin *et al.* 2017 reported the presence of flavonoid in *I. aquatica* (12.71 ± 0.03 mg g-1dwt) following *I. batatus* and *I. carnea* (12.63 ± 0.03 mg g-1dwt) and (11.41 ± 0.05mg g-1dwt) respectively.

Non -Enzymatic Antioxidant Activity

Ipomoea species extracts		Ascorbic acid (mg/g)	Alkaloid (GAE/g)	Tannin (RE/g)	Polyphenol (GAE/g)
I. cairica(L)	Leaf	5.89±4.16	1.7±7.9	1.36±1.22	9.1±6.8
	Stem	8.56±8.02	1.6±6.4	8.73±12.1	1.4±0.64
I. alba(L)	Leaf	1.78±7.81	0.16±0.07	1.38±2.11	6.3±21.7
	Stem	1.99±23.6	2.39±4.0	8.88±22.3	1.04±1.7
I.biloba(L)	Leaf	7.33±2.51	2.4±1.56	1.42±2.51	9.58±2.2
	Stem	2.26±60.3	2.4±8.7	1.34±2.51	1.06±2.0
*I.indica*Burm (Merr)	Leaf	1.7±1.6	3.78±1.72	2.77±0.06	6.8±12.6
	Stem	9.50±14.9	1.7±1.6	3.78±1.72	1.0±1.1
I.hederifolia(L).	Leaf	1.72±12.5	1.29±0.11	5.65±4.59	8.6±2.04
	Stem	2.48±95.6	1.29±0.15	4.43±4.36	1.0±17.7
SED CD(P<0		2.21 1.23	2.16 1.02	1.32 2.03	5.06 2.68

Table. 2 Non-enzymatic antioxidant analysis of phytochemical constituents of methanol extracts of leaves and stems of *Ipomoea* species

Ascorbic acid content

Ascorbic acid expressed the maximum of (9.50±14.9, mg/g), (8.56±8.02 mg/g) in the stem extracts of *I,indica* and *I.cairica*. Among the leaf extracts the highest ascorbic acid content was recorded in *I.biloba* (7.33±2.51mg/g).According to Doka *et al*. 2014 the leaves contained high amount of vitamin C (50 mg/100g). The total phenolic content, expressed as mg gallic acid equivalent (GAE) per 100g sample, showed that *I. aquatic* leaves was rich in phenols (561 mg/GAE/100g). Similarly, Uddin *et al*. 2017 observed the maximum amount of vitamin C content was found to contain in *I.aquatica*(146.66.64mg/100gmdwt) following *I. batatus* and *I. carnea* it was 32.36 ± 0.48mg/100 gm dwt and 121.66 ± 1.85 mg/100 gm dwt, respectively.

Alkaloid content

I.indica has the highest alkaloid content of 3.78±1.72mg/g in the leaf extracts followed by the stem extracts of *I.alba* (2.39±4.0mg/g). In *I.hederifolia* the presence of alkaloid content was on par with the leaf (1.29±0.11 mg /g) and stem extracts(1.29±0.15 mg/g).

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Haraguchi *et al.* 2003 reported that the fresh leaves (2 kg) of *I. carnea* extracted with 50% aqueous EtOH on various cation and anion ion-exchange resins afforded seven alkaloids such as swainsonine(55 mg), 2-epi-lentiginosine(5.7 mg),calystegines B1(13.7 mg), calystegines B2 (41 mg), calystegines B3 (7.8 mg), calystegines C1(25 mg), and N-methyl-trans-4-hydroxy-L-proline(4 mg).

Tannin content

The non- enzymatic antioxidant of tannin expressedthe maximum of 8.8±2.23 RE/g, in the stem extracts of *I.alba* followed by 8.73±1.21RE/g and 3.78±1.72 RE/g in the stem extracts of*I.cairica* and *I.indica* when compared to the leaf extracts. Tannin content showed the maximum in the leaf extracts of *I.hederifolia* (5.65±4.59 RE/g) and *I.biloba* (1.42±2.51 RE/g) when compared to the stem extracts. Essiett and Ukpong, 2014 reported the presence of tannin content of 2.46 mg/100 g, 1.72 mg/100 g and 4.51 mg/100 g in *I. involucrata*, *I. triloba* and *I. batatas* respectively.

Polyphenol content

The polyphenol content of the leaf extracts was found to be the maximum in all the species of *Ipomoea*.The highest content was observed in *I.biloba*(9.5± 2.2 GAE/g) and *I.cairica* 9.1±6.8GAE/g) followed by *I.hederifolia* (8.6±2.04 GAE/g), *I.indica* (6.8±1.26 GAE/g) and *alba* (6.3±2.17 GAE/g). The least value of Polyphenol content was observed in the stem compared to the leaf extracts of all the species. Kumar *et al.* 2014 observed the highest amount of TPC in methanol extract and reported the total phenolic content was significantly higher in *I. pescarpae* leaf as compared to other medicinal marine plant like *Salsola kali* which exhibited 17.23 mg GAE/g DW and in glycophytic species *Nigella sativa* L. which showed 10.04 mg GAE/g DW.

Conclusion

These five studied plants are of equal importance due to the presence of most of the tested major phytoconstituents. Since these plants can be used in the treatment of different ailments, the medicinal roles of these plants could be related to such identified bioactive compounds. Further research should be carried out to exploit the biomedical applications of these five *Ipomoea* species as they possess abundant phytoconstituents for their full utilization.

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Article 8

Comparative Action of Natural Compounds and Azole Drugs on the Clinically Important Fungal Pathogens

Kavitha Dhandapani, Ramya Ravindhiran, Ramya Krishnamurthy, Karthiga Sivarajan and Jothi Nayaki Sekar.

Abstract

Fungal infections are the predominant case for major mortality rates at global level since three decades. The alternative for the treatment and management of fungal infections is contemplated as an alarming issue. The present study has come up with evaluating the antagonistic potential of alkaloid fractions of *Couroupita guianensis* flower, isatin (an alkaloid of *Couroupita guianensis*) and azole drug combination (itraconazole and fluconazole) on the pathogenic fungi. The inhibitory potential of the selected compounds was understood by overlay assay, morphological characterisation of pathogens treated with antifungal compounds and MIC and MFC. The pharmacokinetic profile of selected compounds has proved the good absorption and distribution properties. Molecular interaction of isatin with the virulence proteins of *Candida albicans* such as SAP2 and SAP5 was presumably vital in exerting its antifungal potential. This study has concluded that the isatin is the promising compound for treating aspergillus and candida related infections.

Keywords Fungal infections, Isatin, Virulence proteins, Antagonism, Molecular interactions

1. Introduction

Fungal infections are contemplated as the major alarming threat at global level and reported major mortality rate. Cell wall is the important structure for maintaining the integrity and viability in fungi (Pathakumari *et al*., 2020). More than billion of fungal species were found in the ecosystem where 625 fungal species are approximately known to infect the class of vertebrates and 200 of them causing the infections in human either as commensal bacteria or pathogenic bacteria thus, causing the severe infections in human. The pathogens are posing a big threat to the well being of human and the pathogen shows the increased incidence of systemic and invasive fungal infections, allergy and developing the resistant to particular antifungal compounds. The growing emergence in the infections caused by the filamentous fungi has been associated with major mortality and morbidity rate worldwide (Fisher *et al*., 2020).

The treatment strategy of fungal infections mainly relies upon the use of antifungal therapies and antifungal drugs. Currently, five classes of antifungal drugs can be employed in the treatment of systemic mycotic infections such as polyenes (amphotericin B), azoles (posaconazole, fluconazole, voriconazole, itraconazole and isavuconazole), echinocandins

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(caspofungin, micafungin and anidulafungin), allylamines (terbinafine), antimetabolites flucytosine. The antifungal resistance towards the existing antifungal drugs become a global threat (Gintjee *et al*., 2020).

Due to the antifungal resistance to the existing antifungal drugs, new antifungals have the characteristics of targeted action and inhibitory potentials against the pathogen to be explored. Combinatorial therapy is represented as an efficacious approach to enhance the effectiveness of antimicrobial therapy to combat the antifungal resistance (Gucwa *et al*., 2018; Widerhold, 2017; Li *et al*., 2016).

Itraconazole is an antifungal drug belongs to the category of triazole derivative having wide range of antifungal activity. It is well absorbed in the host and easily permeable across the blood-brain barrier into the cerebrospinal fluid. The half-life of this medication in plasma is about 30 hours. Liver is the major metabolizing organ where it get easily metabolized and eliminated via urine. Fluconazole is also a triazole derivative with a huge application as an antifungal drug. Like itraconazole, the drug is easily metabolized and readily across the blood-brain barrier to produce its targeted action (Li *et al*., 2019).

Apart from the combinatorial therapy, the medicinal plants contain many bioactive compounds which make the medicinal plant and their products in to pharmaceutically important. Traditional medicine endures the linchpin for treating and nurturing the health of the individuals in various developing countries (Sheba and Anuradha, 2019).

Couroupita guianensis is one such medicinal plant has plenty of secondary metabolites having various reported properties like, antimicrobial, antioxidant, anti-diabetic, anti-inflammatory and anti-cancer properties. All most all parts of this plant effectively involved to contributes several medicinal properties (Keerthana *et al*., 2018). The tree, *Couroupita guianensis* is generally referred as Cannon ball tree in English, Nagalingam tree in Tamil, which is a large deciduous tropical tree (Shah *et al*., 2012; Sumathi and Anuradha, 2017). The leaves are up to 6 inches in length and are considered as resources of folk medicines. It has flowers in racemes which are in yellow, reddish and pink in colour with stunning fragment. The flower has a significant special in Buddhist period due to the presence of Lord Shiva shape at the center and snake shaped pollen with good fragrance (Sheba and Anuradha, 2019).

Hence, the present study has focused to evaluate the antifungal activity of natural compounds and naturally derived compounds against wide range of human fungal pathogens.

2. Materials and Methods

2.1. Collection and preparation of sample

The flowers of *Couroupita guianensis* was procured from Perur temple, Coimbatore and the plant specimens were identified, certified and the voucher specimen number (2430)

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was deposited at the Botanical Survey of India, Southern Circle, Coimbatore. The flower sample was rinsed thoroughly two to three times with running tap water and was chopped into small pieces. Shade dried the flower sample at room temperature for 2 days and the powder was prepared after the sample was completely dried. 5g of dried powder was used for the preparation of alkaloid fractions. Fig 2.1. depicts the flower of *Couroupita guainensis*.

2.1. Preparation of alkaloid fraction

(Harborne, 1973)

Fresh flowers (5 grams) were crushed using mortar and pestle by adding 10% acetic acid in ethanol (200ml) and incubated at dark for 4 hours. The extract was filtered after incubation period and the solution were kept in boiling water bath for the concentration reduced to ¼th of its original volume. 25% ammonium hydroxide or 25% ammonia was added to the extract until a precipitate was obtained and then centrifuged at 2500 rpm for 5 minutes. 1% NH4OH solution was added to the residue and filtered. The alkaloid content in the obtained residue was weighed, dissolved in ethanol solution and stored at 4°C until further use.

2.3. Fungal strains collection and maintenance

Pathogenic *Candida albicans and Aspergillus fumigatus* were procured from PSGIMS, Coimbatore. *Aspergillus niger* was isolated from onion. All the fungal strains were grown on potato dextrose agar (PDA) incubated 23ºC-28ºC in dark at dark for 3-4 days. The culture plates were stored at 4ºC until further use.

2.4. Preparation of fungal spore suspension

Candida albicans, Aspergillus fumigatus and *Aspergillus niger* plates were flooded with 10 ml of sterile distilled water and 1% tween 20 solution to dislodge hyphae. This solution with spores were filtered with Whatman no 1 filter paper and the hyphal spores were removed. The prepared spore suspension was stored at 4ºC in the refrigerator and this spore suspension was used for the further assays.

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2.5. Overlay assay

The inhibitory potential of compounds namely, *Couroupita guianensis* flower alkaloid fractions, Isatin and combination of azole drugs (Fluconazole and Itraconazole) on the selected pathogens was studied by overlay method. 200µl of the selected antifungal compounds was poured into the medium containing potato dextrose agar and 100µl of the fungal pathogens was placed on the agar medium. The plates were incubated at dark conditions for 48 hours. After the incubation period, the plates were observed for the growth of fungal pathogens in the presence of antifungal compounds.

2.6. Morphological changes of antifungal compounds treated fungal pathogens

The inhibitory potential of selected antifungal compounds on the fungal pathogens, *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger* was further confirmed by the staining of inhibitory zone obtained in the overlay assay using lactophenol cotton blue. On a clean slide a zone of inhibitory portion was taken and placed a drop of lactophenol cotton blue stain on the top. A sterile coverslip was covered without making any bubbles. The slide was examined at 40 X magnification for observing the antagonism.

2.7. Minimum inhibitory concentration assay

Minimal inhibitory concentration (MIC) was determined based on the 2-fold broth dilution method. The *C. guianensis* flower alkaloid fraction, Isatin and Standard drugs in the concentration ranging from 1000µg/ml to 62.5µg/ml, respectively for each antifungal compound were prepared. 100µl of Sabouraud dextrose broth was pipetted out into the 96 well plate (1st well to 5th well) and 100 µl of each sample containing the desired concentration was introduced into the first well. Then 100 µl from the first well was serially diluted to the final well and 100µl of pathogenic fungi was added to each well. with Pathogenic fungal culture alone served as control. The plate was incubated at dark for 36 hours and the inhibition was noted on next day by observing the visible turbidity. After 36hour incubation, 20µl of 0.5TTC (Triphenyl Tetrazolium Chloride) was added to each well and incubated for 12 hours. MIC was referred as the lowest concentration of each sample that inhibits the visible growth of the fungi. The assay was performed in triplicates.

2.8.Minimum Fungicidal Concentration

In order to determine the minimum fungicidal concentration (MFC), 10 µl of each of the culture with no visible growth after the incubation period was subculture on the surface of the Potato Dextrose Agar (PDA) plates (freshly prepared). The plates were incubated at 37°C for 48 hours. The MFC was documented as the lowest concentration of the selected sample that shows complete inhibition of visual growth of fungal colonies on the agar plate after respective incubation period.

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2.9. Cytotoxicity assay

Cytotoxicity assay was performed to determine the toxicity level of antifungal compounds. It was evaluated by analyzing the hemolytic properties of the antifungal compounds present in the alkaloid fractions of *Couroupita guianensis*, isatin and azole durgsusing goat red blood cells. To assess the cytotoxicity of antifungal compounds, hemolytic activity was performed to evaluate the percentage hemolysis using 4% suspension of goat red blood cells (gRBCs) treated with alkaloid fractions of *Couroupita guianensis*, isatin and azole durgs. The gRBCs were washed thrice with a phosphate buffered saline (PBS).100 µl aliquots of gRBCs suspension were taken into 96 well microtitre plates, and then 100 µl of alkaloid fractions of *Couroupita guianensis*, isatin and azole durgsin PBS was added into each well. The plates were incubated at 37°C for 1h. Then the mixture was centrifuged at 1500rpm for 10 minutes, and aliquots with PBS and 100% with 0.1% Triton X-100.The following equation is applied for the calculation of percentage hemolysis.

$$\text{Percentage hemolysis (\%)} = \frac{\text{(Abs414nm in the compound solution- Abs414nm in PBS)}}{\text{(Abs414nm in0.1\% Triton x100-Abs414nm in PBS)}} \times 100$$

2.10. Selection of target proteins

The major virulence protein of the *Candida albicans* involved in the infectious process in human is secretion of aspartic proteases (SAPs) which known to damage the tissue barriers, host defense molecules and cleaves the proteins required for nutrient supply. Based on the function of SAPs in different times of infection process and during different types of infection has been classified into 10 different SAP genes. The genes namely, SAP1, SAP2 and SAP3 are involved in tissue damage, cutaneous epidermis and invasion of oral epithelium. In systemic infections SAP4, SAP5 and SAP6 genes are involved. Therefore, SAP2 and SAP5 were selected as target of *Candida albicans* to be docked with the alkaloid compound (isatin) and PDB ID 1EAG for SAP2 and 2QZX for SAP5.

2.11. Preparation of target protein

The target proteins of *Candida albicans* were prepared by Protein Preparation Wizard in Schrodinger window which accepts a protein from its raw state to a state in which it is properly prepared for calculations. After optimization and minimization, the refined target proteins of *Candida albicans* were prepared and the results were saved in the format of .png.

2.12. Preparation of ligands

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Isatin, the potential alkaloid was selected for the molecular interactions with the target proteins of *Candida albicans*. The structures of these ligands were downloaded from NCBI-PubChem database (http://www.ncbi.nlm.nih.gov/pubchemcompound) and were saved in a Word document. The structure of the selected compound was drawn by the tools in the Maestro window of Schrödinger. In a project table the new entries of the refined structures were entered. The ligands were prepared using the application, LigPrep 2.1, a module on the Maestro window of Schrödinger. LigPrep generates a number of structures for each structure of the ligand (inserted) with various tautomers, ionization states, stereochemistry and ring conformations and removes compounds based on different criteria such as molecular weightand types of functional group it possesses. The prepared ligands were subjected for docking with the target proteins.

2.13. ADME Predictions of isatin

The QikProp 3.0 module has been used for the prediction of physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. Secondly, predicting molecular properties, it provides ranges for comparing a particular molecule's properties with those of 95% of known drugs. By employing the QikProp 3.0 module of Schrödinger, the various pharmacokinetic properties including, Absorption, Distribution, Metabolism and Excretion (ADME) were predicted.

2.14. Molecular interaction of isatin with target proteins of *Candida albicans*

Glide applies a hierarchical series of filters to search for possible locations of the ligand in the active-site region of the receptor. The receptor grid was generated to dock with the ligand molecules. Glide 4.5 module of Schrödinger was run to perform the docking of ligands with the target proteins of *Candida albcans*. Standard Precision Mode (SP) was performed for molecular interactions of compounds and virulence proteins. The interacted virulence protein and the ligands were viewed with Glide Pose Viewer on maestro Schrödinger window. The best docked images of ligands with the target proteins were saved in the format of .jpg files.

3. Results and Discussion

3.1. Quantification of alkaloids in the alkaloid fractions of *Couroupita guianensis* flower

The alkaloid fraction of *Couroupita guianensis* flower was screened for its antimicrobial and pharmocological activities. Hence, the *Couroupita guianensis* flower was crushed and precipitate the alkaloids in the flower extract using the concentrated ammonium hydroxide. The prepared alkaloid fraction was quantified for its alkaloid content and the results exhibited that the amount of alkaloids present in the alkaloid fractions of *Couroupita*

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guainensis flower extract was found to be 2.42 mg/ml. Fig. 3.1 showing the prepared alkaloid extracts of *Couroupita guianensis*.

Figure 3.1: Alkaloid extract of *C. guianensis* flower

3.2. Antagonistic potential of alkaloid extracts, Isatin and drug combinations on selected pathogens

Figure 3.2. depicts the inhibition of selected fungal pathogen by the alkaloid extracts, Isatin and azole drug combinations (Fluconazole and Itraconazole). All the candidates showed good inhibitory profile on each pathogen namely, *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger*. Several scientific evidences showed that the inhibitory potential of natural compounds effectively killthepathogenic fungi (Sanchez *et al*., 2016; Medeiros *et al*., 2016; Matar*etal*.,2017).

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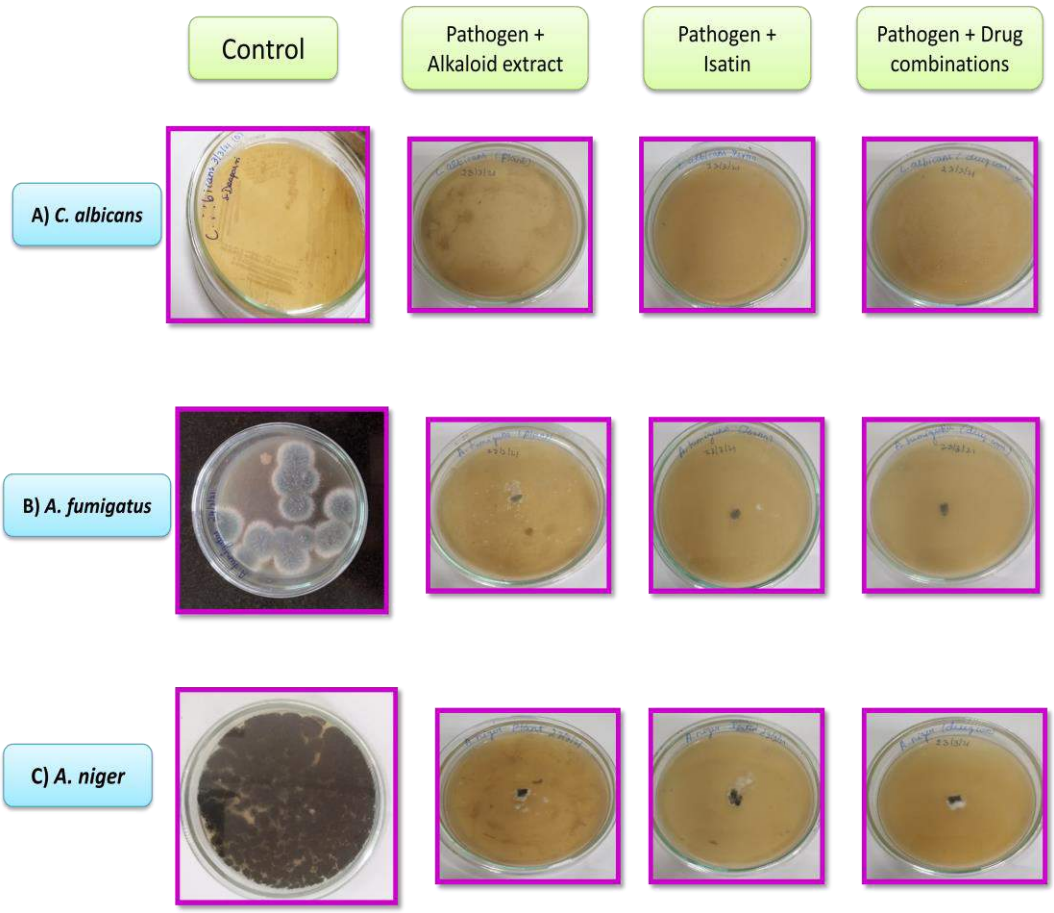

Figure 3.2: Inhibitory potential of alkaloid fractions of *C. guianensis*, Isatin & standard drugs on A) *C.albicans* B) *A.fumigatus* C) *A.niger*

3.3.Morphological changes of fungal pathogens treated with antifungal compounds

The hyphal projection is the key for infectious process of any pathogen. The inhibitory potential of the compounds namely, *Couroupita guianensis* alkaloid fraction, isatin and combination of drug against the fungal pathogens were further proved by performing the lactophenol cotton blue staining (Figure 3.3). The hypha of the fungal pathogen was damaged due to the antagonistic potential of *Couroupita guianensis* alkaloid fraction, Isatin and combination of drug (Fluconazole and Itraconazole). The irregular and collapsed hyphae were observed in the pathogens treated with the selected antifungal compounds while the good morphology with the extended hyphae was noticed in the control plate inoculated with pathogen alone. All the compounds namely *Couroupita guianensis* alkaloid fraction, Isatin and combination of drug (Fluconazole and Itraconazole) have the potential to inhibit the growth of fungal pathogens.

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3.4.Minimum Inhibitory Concentration and Minimum Fungicidal Concentration

The minimum concentration of selected compounds namely, *Couroupita guianensis* alkaloid fraction, Isatin and combination of drug (Fluconazole and Itraconazole) were determined against fungal pathogens (Table 3.1). The best concentration to inhibit the growth of fungi by the alkaloid fraction of *Couroupita guianensis* was found to be 250 µg/ml for *Candida albicans* and *Aspergillus niger*. The 500 µg/ml of the alkaloid fractions was observed as MIC for *Aspergillus fumigatus*. The MIC of isatin was found be effective at 250 µg/ml for the inhibition of all the selected human fungal pathogens except 125 µg/ml for *Candida albicans*. The combination of standard drugs namely, fluconazole and itraconazole was found to be effective in killing the *Aspergillus fumigatus* and *Aspergillus niger* at 500 µg/ml and *Candida albicans* at 250 µg/ml.

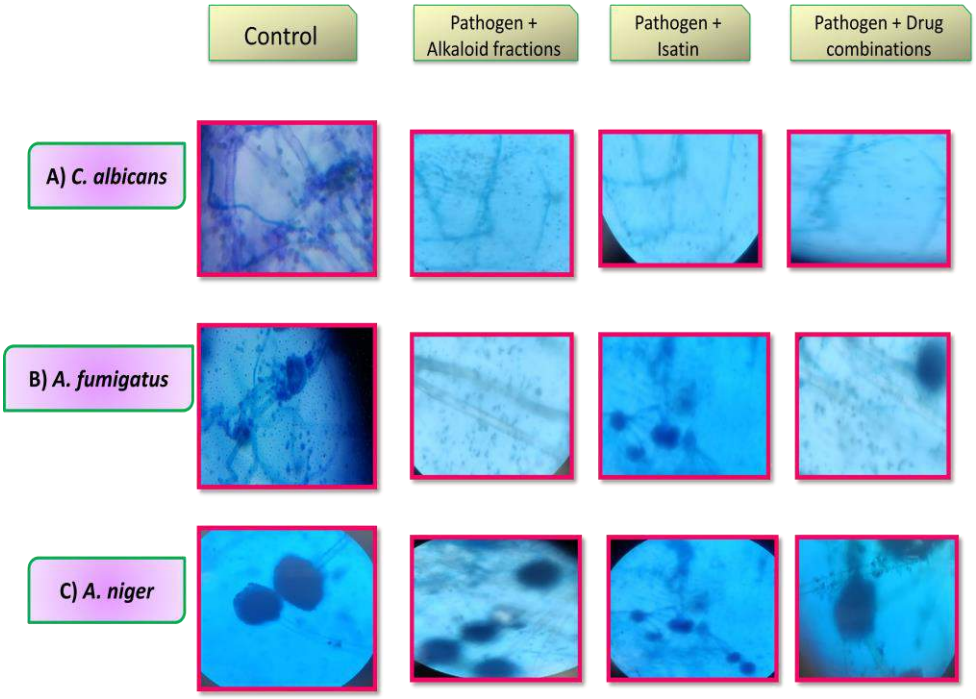

Figure 3.3: Morphological changes of A) *C.albicans* B) *A.fumigatus* C) *A.niger* treated with antifungal compounds

Figure 3.4. explains the MFC of selected compounds and antifungal drugs on the fungal pathogens namely, *Candida alicans*, *Aspergillus fumigatus* and *Aspergillus niger*.The minimum fungicidal concentration of alkaloid flower extract was found to be 500 µg/ml for the complete killing of the pathogen, *Aspergillus niger*. The *Aspergillus fumigatus* was completely killed at 500 µg/ml of drug combination and the 250 µg/ml of isatin were found to be effective in killing of *Candida albicans*. The minimum inhibitory and minimum

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fungicidal concentration of antibiotics and antimicrobial compounds to completely kill the growth of microbial pathogens was reported in many studies (Sanchez *et al*., 2016; Gucwa *et al*., 2018).

Name of the Sample	**Concentration of sample in µg/ml**	**Pathogenic fungi**		
		Candida albicans	***Aspergillus fumigatus***	***Aspergillus niger***
Couroupita guianensis alkaloid fraction	1000	-	-	-
	500	-	-	-
	250	-	+	-
	125	+	+	+
	62.5	+	+	+
Isatin	1000	-	-	-
	500	-	-	-
	250	-	-	-
	125	-	+	+
	62.5	+	+	+
combination of drug (Fluconazole and Itraconazole)	1000	-	-	-
	500	-	-	-
	250	-	+	+
	125	+	+	+
	62.5	+	+	+

"+" indicates the growth of pathogen in the presence of antifungal compounds

"-" indicates the inhibition of growth of pathogen by the antifungal compounds

Table 3.1: Minimum Inhibitory Concentration(MIC) of selected compounds on candida and aspergillus spp.

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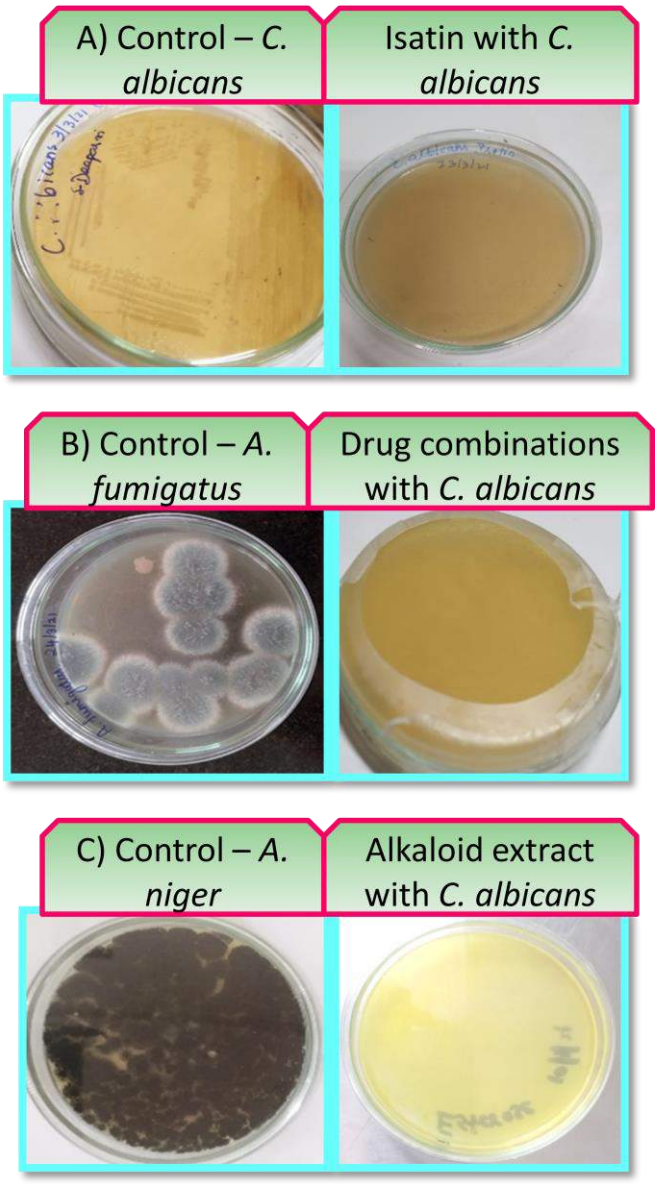

Figure 3.4: Minimum Fungicidal Concentration (MFC) of selected antifungal compounds on pathogenic fungi

3.5. Cytotoxicity assay using erythrocytes

Cytotoxicity assay was performed to find out the toxicity level of antimicrobial compounds for their safer use. The hemolytic assay was done with alkaloid fractions of *Couroupita guainensis*, isatin and drug combinations (fluconazole and itaconazole) using goat red blood cells (Figure 3.5). Percentage of hemolysis was increased with the concentration of samples such as, alkaloid fractions of *Couroupita guainensis*, isatin and drugcombinations (fluconazole and itaconazole). Hemolysis was occurred in unnoticeable amount in the selected concentration of samples used. In this context, the study has proved

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that the selected compounds can be used for treating fungal infections and related diseases due to its efficacy and safer use with minimal side effects.

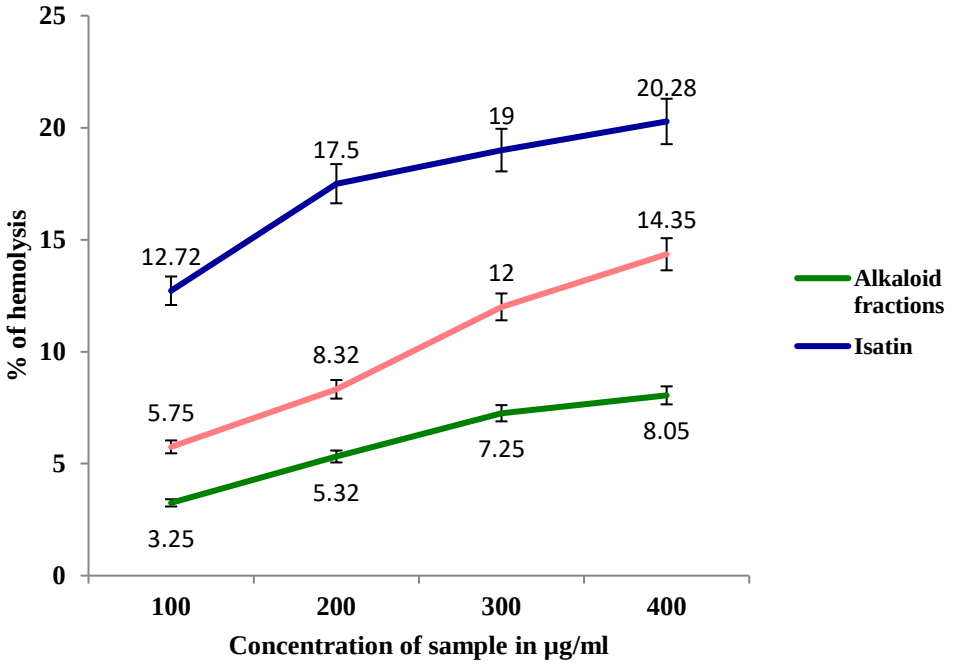

Figure 3.5: Cytotoxicity of Alkaloid fraction of flower, Isatin, Azole drug combinations (Itaconazole & Fluconazole)

3.6. Bioavailability profile of isatin

For the discovery of a potential drug, a molecule/compound should possess the good pharmacokinetic profile (ADME). The minimum concentration of isatin is effective to completely kill *C. albicans* while compared with the other two fungi. Therefore, molecular interactions of isatin with the target proteins of *C.albicans* were studied. Table 3.2 explains the QikProp results of the isatin compound. The QikProp results indicating that isatin satisfy the Lipinski's rule of five (refinement of drug likeness) and is used to predict the compounds have biological or pharmacological activity as orally active drugs in human. The percentageof human oral absorption in gastrointestinal tract is 74 for isatin. The lipophilicity of the compound is expressed as the partition co-efficient P in octanol/water and indicating that isatin is lipophilic thus, expressing good absorption and distribution profile. ADME predictions are being an important property for the successful drug discovery and several scientific studies supports the method for pharmacokinetic prediction (Bouchal *et al*., 2019; James *et al*., 2018).

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S.No	Descriptors	Standard value	Isatin Value
1	Molecular weight (Da)	130.0-725.0	147.13
2	Number of hydrogen bond donors	0.0/6.0	1.000
3	Number of hydrogen bond acceptors	2.0-20.0	4.500
4	QP log P for octanol/ water	-2.0/6.5	0.138
5	Apparent Caco-2 Permeability (nm/sec)	<25 poor, >500 great	373
6	Apparent MDCK Permeability (nm/sec)	<25 poor, >500 Great	170
7	Lipinski Rule of 5 Violations	Maximum is 4	0
8	% Human Oral Absorption in GI (±20%)	<25% is poor	74%
9	Qualitative Model for Human Oral Absorption	>80% is high	Medium

Table 3.2: ADME prediction of Isatin by QikProp 3.0

3.7.Molecular interactions of isatin with the virulence proteins of *Candida albicans*

Table 3.3 represents the binding potential of isatin with the target proteins of *Candida albicans* such as SAP2 and SAP5. The docking efficiency and molecular interactions exhibiting good hydrogen bond interactions and good contacts of the isatin with the pathogenic proteins is depicted in figure 3.6. The glide score for the interactions with virulence proteins involved in the infectious process is -5.79 for SAP2 and -5.56 for SAP5. Several scientific evidences support the molecular docking studies for the drug development and drug discovery (Castillo *et al*., 2020; Ndaba *et al*., 2020; Sari and Kart, 2020). The isatin was found to possess good interaction efficiency with the target virulence proteins of *Candida albicans* revealing its importance as drug candidate to treat *Candida albicans* associated infections.

Interactions parameter	SAP2	SAP5
Glide Score	-5.79	-5.56
Energy (Kcal/ Mol)	-22.71	-25.54
Good VDW	84	96
Pose number	103	79
Conformation number	1	1
No.of H-bonds	2	1

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Protein residue atom	A:THR222:(H)H A:GLY220:(O)O	A:GLY220: (O)O
Ligand atom	14(O) 1(H)	1(H)
H-bond distance (Å)	2.365 1.872	2.128

Table 3.3: Molecular interactions of isatin with virulence proteins SAP2 and SAP5 of *Candida albicans*

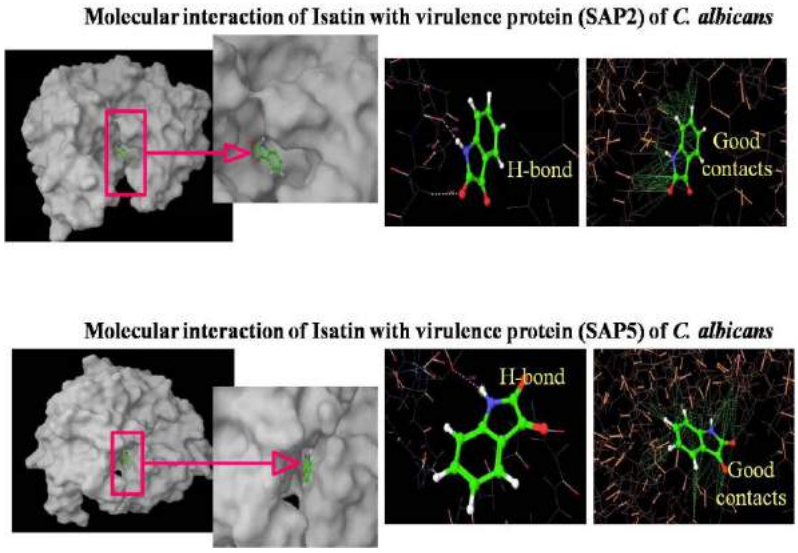

Figure 3.6: Molecular interaction of Isatin with virulence proteins (SAP2 & SAP5) of *C. albicans*

Conclusion

Owing to the limited applications of existing antifungal drugs, an urge for the development of novel antifungal compounds from the natural sources attained much importance. The antifungal assays explained the antagonistic nature of the selected natural compounds and drugs towards the pathogenic fungi. The pharmacokinetic properties and molecular interactions of isatin pave the way for the development of antifungal drugs. Hence, this study has proved that the selected compounds can be employed for treating fungal infections and related diseases due to its efficacy and safer use with minimal side effects. These selected compounds may act as a lead compound for the development of novel drugs by the pharmaceutical industries to treat fungal infections.

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Article 9

Impact of Covid-19 Pandemic on Fisheries

Reshma Jain

Abstract

In India, COVID-19 has proved to be a very dangerous disease for the economy as well as the health system. Breakdown of demand and supply chain of agricultural product such as fish and fishery products for the nation-wide and lockdown will directly affect 14.5 million people. COVID-19 disease has caused many different impacts in production, distribution and marketing, inland fisheries, marine capture fisheries, seed supply and sea food export etc. The whole economy of the world is disturbed, while many developed countries are trying to cope up with this novel challenge to restore the economy and regulate the proper demand and supply within their nations. Many countries are providing helping hands to India to battle this covid war through vaccination and in improving the health industry sector. However, it is also necessary to focus on the employment and revival of primary and small sectors like agriculture, weaving, fisheries, etc. Immediate response of government to tackle covid is lockdown but it's side effects like increased unemployment, increase in poverty, closing of markets, loss of jobs has led to shift in the state of middle class to lower class in the earning category which mostly consists of small farmers, fish farmers, weavers, etc. Also, the indirect impact on food and nutrition system has challenged millions of people who consider aquatic foods as major source of protein. Thus, this project mainly focuses on the study of the impact of COVID-19 on the fisheries sector and aims to give few suggestions to set a strategy plan to improve this pandemic shock.

Keywords COVID-19, economic downturn, fish farming, supply chain, sea food export.

1. Introduction

Starting from Wuhan city, china, on December 31 2019, due to COVID-19 disease. There is a bad impact on the whole World. By observing the very unpleasant extent of the outbreak, the World Health Organization (WHO) declared COVID-19 as a global emergency on January 30 2020. But as the disease is highly transmissible which spread from one person to other person and vaccine has not been developed, we can take action to slow the spread of this virus by self-quarantine, social distancing, travel restriction, and close down of different public facilities, transportation and even lockdown of the entire country.

The death rate is 3.5% globally and it is very harmful for us. At the time of writing this article there were 3.7 million confirmed cases with 2 lacks sixty-five thousand deaths reported from 213 countries and territories throughout the world, and India was with 53

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thousand confirmed cases and 1700 of deaths. Thus, in order to control the spread the government of India imposed a 3-week long lockdown nationwide on March 24, 2020.

The government of India spends only 1.5 percent of the total GDP on public health so the health system of the country is weaker in comparison to other countries. But a COVID-19 case in India was increasing so the government extended the lockdown up to May 3, and expanded 17 may. Country is facing great loss from this COVID-19 pandemic. A report of the United Nation indicating more than four hundred million people of the informed sector (self-employment) in India May sink into poverty.

India is the second largest fish producing country in the world with 6.5 percent of global production. 14.5 million People are engaged in fisheries in India. So, in this situation, an attempt was made to examine the potential impact of COVID-19 pandemic on the fisheries sector in India. And various strategies were implemented for the control of the COVID-19 pandemic. It will also insure the speedy recovery of the sector.

COVID-19 has proved to be very dangerous disease for the economy as well as the health system. Due to COVID-19 disease whole world is in the lockdown due to this supply chain of agricultural product and lockdown will directly affect 14.5 million people. Due to this condition the whole economy of the world is disturbed. Many countries are providing helping hands to India to battle this COVID war through vaccination and in improving the health industry sector. Immediate response of government to tackle COVID is lockdown but its side effect like increased unemployment, increase in poverty, closing of markets, loss of jobs has led to shift in the state of middle class to lower class in the learning category which mostly consists of small farmers, fish farmers, weaves etc.

1.2.Indian Fisheries before COVID-19 Period

The COVID-19 shock is playing out in almost a similar manner all around the world due to this there is slowdown in demand and supply due to this as a result of economic slowdown. In India the COVID-19 period may be long lasting because before the COVID-19 period also the Indian economy was not too good. In 2018-19 India fish production was about 6 percent greater than previous year. Sea food is the fourth biggest exporter in the world. In 2018 India exported 13, 77,244 tonnes of seafood. In 2017-18 Andhra Pradesh was the leading fish producing state in the country, followed by west Bengal and Gujarat by producing 34.5, 17.42 and 8.35 lakh tonnes fish respectively. Gujarat was the first state in marine production in the country.

Some large and important marine fish were hilsa, Indian oil sardine, sea catfish, lizard fish, seer fish, barracudas, silver bellies, anchovies, clupeids, mackerels, skipjack tuna, yellowfin tuna, crust crab and penaeid and non-penaeid prawn. Indian large carp’s fish were the most cultured species followed by exotic carps, cat fishes, minor carps and murrel

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fishes. 78 percent fish was used as fresh marketing, followed by 8 percent as freezing and 4 percent as curing.

1.3.Impact

Fish are one of the major nutrition's parts of the global food chain and key source of employment generation among rural coastal people. Fish are one of the most traded food products throughout the world. Decreased consumer demand, lower supplies, and interference in supply chains will directly affect the people engaged with industry such as fisherman, fish processor, fish vendors, suppliers and transport workers. Postponement of various research and development programmes, science and management meetings will stop the growth of this sector. Fishing ban season started from July 15 and will continue till June 15 in the last coast. For the west coast it is from June 1 to July 31. As a result, marine fishermen will not be able to fish for about 75 days in the last coast and 130 days in the west coast. So, there is no doubt how this pandemic is going to destroy (damage) the fishing industry even after the lockdown.

Rural agriculture labour payment was very low which made them unhappy both in nominal and real terms from the pre COVID-19 period. In this particular situation the corona outbreak will stop the fishery activity and supply through several dimensions, Lake Crop harvesting, processing, procurement (in obtaining good services) and marketing. Problems in transportation, restriction in labour movement will directly affect the farming and processing industry. March to June is the best season for fish farming as well as it is the best season for shrimp fish farming but this will get difficult because of non-availability of workers. Shortage of fish seed fertilizer and other inputs may also decrease the production level. Suppliers and producers will be greatly affected by reduced demand, transport limits and closure of different restaurants. Due to lack of proper functioning of supply chains, lots of farmers are not able to sell their crops properly. This causes very big losses for farmers. Though fish and food supply chains are very necessary still fish and food supply chains are facing a lot of difficulties in marketing. Some people are not buying the fish in fear of getting affected. Japan, US are buying selectively but European market is totally closed off. Thus, the slow farming sectors are going to put the fishery industry of the country in a severe difficulty.

1.4.Mitigation Measures

Poor sections of any society are very weak to face any event that causes harm ex. this COVID-19 situation. India is known as an agricultural backbone. Country with 85 percent farmers in which maximum are landless and are labours. So, the welfare measures must be taken by both the state and central government to protect them from COVID shock.

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During and on the lockdown every farmer, agricultural labour, workers have to be protected from health and financial damage.

Proper awareness during farming should but don't like social distancing in harvesting, marketing, packaging, proper sanitization, personal care and wearing face masks. It will help to prevent the spread of the COVID-19.

The products which are not long lasting will get spoiled after a few days. Like fish etc. need to continue in the market and the government should develop a minimum support price for fish and fishery products which will be helpful for farmers as well as for consumer point of view.

As our country is going through a lockdown since March 24. Thus, the government should consider some alternative means to reduce the ban period, particularly for this year or special financial assistance may be provided to the fisher communities.

At present out of 140 million beneficiary farmers only 84.6 million are under the PM-KISAN scheme, thus the government should increase the inclusion as well as the number of instalments to protect the profession and community.

On the other hand, the government should provide a tax-free helpline. For the farmers, solving queries related to farming and marketing.

The loan which was taken by the farmers from bank like agricultural loans should be expanded and facilitated for smooth flow of credit for the farmers.

Block chain technology should be included in the seafood sector which could decrease the pain of the global seafood industry.

Moreover, a separate complete financial package along with detailed guidelines must be developed for the aquaculture and capture fisheries sector of the country for protecting the industry and connected population from the very great effect of the pandemic.

It will also ensure the speedy recovery of the sector.

2. Result and Discussion

In India, COVID-19 has proved to be a very dangerous disease for the economy as well as the health system. Breakdown of demand and supply chain of agricultural product such as fish and fishery products for the nationwide and lockdown will directly affect 14.5 million people.

Due to COVID-19 disease the whole economy of the world is disturbed. It is also necessary to focus on the employment and revival of primary and small sectors like agriculture, weaving, fisheries etc.

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Thus, this project mainly focuses on the study of the impact of COVID-19 on the fisheries sector and aims to give few suggestions to set a strategy plan to improve this pandemic shock.

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Conclusion

COVID-19 outbreak has been a global health emergency, and it has a great impact on developing countries like in India. With a 1.3 billion population and the dangerous situation of the economy in the pre-COVID period, a long-time lockdown would be a very serious problem for the economy especially for the agriculture sector which is already in a weak condition. India, with its apt governance, took the situation as a challenge and did in all ways possible to overcome this pandemic situation. At the same time the government and policy makers need to be prepared to minimize the impact of the shock and economic depression of the economy in the post and period.

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Article 10

Comparative Study of Modular Multilevel Inverter with Various Modulation Techniques

Manimala.P and Sujatha Balaraman

Abstract

Modular Multilevel Inverter (MMI) is one of the most promising multilevel inverter topology for high-power and high-voltage applications. This paper presents performance analysis of Modular Multilevel Inverter with different modulation techniques for various levels. The modulation techniques used for chopper cell based MMI are reviewed and applied for controlling the output voltage waveform without adding any complexity to the power circuit. In this study, the functionality of Half-bridge MMI was studied using modulation techniques such as Multi-Carrier based Pulse Width Modulation (MCPWM) and Nearest Level Control Modulation respectively. The simulation is carried out in MATLAB/ Simulink. It is found that the carrier based Phase Position PWM (PDPWM) technique yields lesser Total Harmonic Distortion (THD)of the load current and voltage. However, the PDPWM technique requires carrier signals of high switching frequency and involves high switching losses. In order to reduce the power loss and to improve the power quality,an attempt has been made withNearest Level Modulation (NLM) based PWM technique which can operate with fundamental switching frequency. From the simulation results it is evident that NLMPWM gives optimal performance than PDPWM.

Keywords Modular Multilevel Inverter, Multicarrier PWM techniques, Total Harmonic Distortion, Nearest Level Modulation.

1. Introduction

In recent years multilevel inverters are attaining much interest in electrical power industry. They are appropriate for high voltage - high power applications. Multilevel inverter yields a staircase output waveform, which resembles a pure sinusoidal wave. The resultant voltage of the multilevel inverter has reduced number of harmonics when compared to the conventional two level inverter's output voltage. Neutral-Point-Clamped Inverter or diode-clamped inverter, Flying Capacitor (FC) inverter and Cascaded H-bridge (CHB) Inverter are some of the most common multilevel inverter topologies. In a diode-clamped inverter, balancing the capacitor voltage remains as a critical problem in high levels [1-2]. When the number of levels increases, the number of power electronic devices gradually increases and the realization becomes difficult [3].

In case of FC inverter, the complex control circuit is required to maintain the voltage levels for all of the capacitors. Compared to the diode-clamped inverters, a large

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number of capacitors are needed for FC inverter. Due to these demerits, they become less attractive for higher levels [4].The CHB topology is more suitable than other conventional MLIs due to the modular structure, but it requires large number of isolated dc sources that are generally given by a bulky, multi-winding transformer along with rectifiers and high numbers of power electronic devices [5]. In [6], a novel Improved Firefly Algorithm forbalancing the Three-Level Neutral Point clamped inverter was proposed. An improved firefly algorithm was realistic for computing optimal switching angles for programmed PWM permitted to eliminate the pre specified lower order harmonics and to attain the desired fundamental voltage of multilevel inverter [7].

The Modular Multilevel Inverter was proposed to overcome the drawbacks associated with conventional multilevel inverters. Some of the features of MMI are modular structure, simple realization and redundancy.Theinverter has been built up by modules and it can be easily scaled to different voltage and power levels so that it has a modular realization and also the harmonic distortion reduces as the number of voltage level increases. The converter can continue to operate even though some of components experience failure. Also, it is better in managing the fault [8]. The modular construction of MMI reduces investment and life cycle cost with the use of standard components [9]. Pulse Width Modulation technique has the ability to control the fundamental voltage across the load. The waveform quality of the inverters is decided by PWM techniques. Therefore, it is very much important to choose the appropriate PWM method. The main functions of the PWM techniques are to approximate the reference vector, to determine the switching sequence, to determine the switching states and to determine the duty cycles. The PWM methods are classified into Selective Harmonic Elimination PWM (SHE-PWM), Space vector PWM and Carrier based PWM, Nearest Level Control Modulation. In this paper the simulation of various PWM techniques are considered for different levels of MMI.

The outline of this paper is as follows. In Section II, the concept and structure of MMI with its operating principles are presented. Control and Pulse Width Modulation Technique and its types are described in Section III. Comparison of harmonic performance in various level of MMI is presented in section IV. The conclusion and reference sections follow.

1.1 . Structure of Modular Multilevel Inverter

Figure 1 shows the typical structure of MMI. Each leg consists of an upper and lower stack of cells which is called as arms. Each arm consists of n-1 cascade connection of sub modules(SM), where n is the number of level. The half Bridge SM in figure 1(b) has been used to model the MMI. Each SM is a simple chopper cell which encompasses two semiconductor devices, S_m (main switch) and S_a (Supplementary switch), two antiparallel diodes Dm and Da, and a capacitor C. An arm inductance is used to protect the power electronic switches from the inrush current caused by the capacitors as well as to limit the

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circulating current in normal operation [10-12]. The upper and lower arm is connected with arm inductance. As the circulating current is produced during the operation, an arm inductance needs to be integrated so that the upper and lower arms get isolated. [13]. By switching the SMs on and off at the precise time, the voltage can be built up stepwise, producing nearly a sinusoidal shaped output voltage or current.

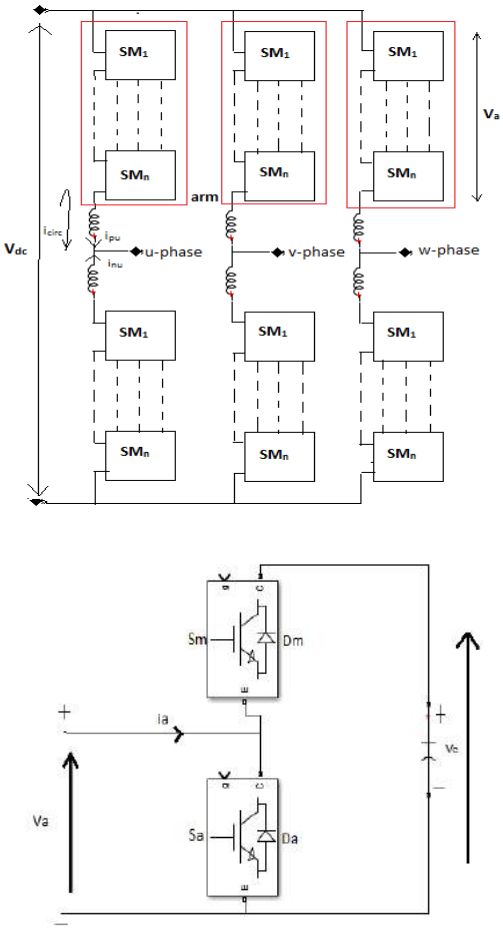

Fig.1. (a) Structure of Three Phase Modular Multilevel Inverter (b) Half Bridge Sub module

The arrangement of the sub-modules is shown in figure 1. When the main switch (S_m) is switched ON and the supplementary switch (S_a) is switched OFF, the voltage of the capacitor Vc is applied to the SM terminals. The arm current i_{arm} is positive and the capacitor will charge; while an arm current i_{arm} is negative, the capacitor will discharge. When main switch (S_m) is turned OFF and auxiliary switch (S_a) is turned ON, the capacitor is bypassed, making sure the terminal voltage, Va is zero. When both switches S_m and S_a turned off, the positive current, i_{arm}, will charge the capacitor, C, while a negative i_{arm} will bypass the capacitor.

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For ‘n’ level MMI requires n-1 switching modules. The five level MMI uses 6 arms and in each arm there are four switching modules. A switching module consists of two switches namely main switch refers to (S1, S2, S3…..S8) and the supplementary switch refers to (S1’, S2’, S3’…S8’). The supplementary switch is in series with a capacitor and this series arrangement is in shunt across the main switch.

OUTPUT VOLTAGE LEVEL	ON STATE SWITCHES		OFF STATE SWITCHES	
	UPPER MODULE	LOWER MODULE	UPPER MODULE	LOWER MODULE
Vdc/2	S1,S2,S3,S4	S5’,S6’,S7’,S8’	S5,S6,S7,S8	S1’,S2’,S3’,S4’
Vdc/4	S2,S3,S4,S5	S4’,S5’,S6’,S7’	S1,S6,S7,S8	S1’,S2’,S3’S8’
0	S3,S4,S5,S6	S3’,S4’,S5’,S6’	S1,S2,S7,S8	S1’,S2’,S7’,S8’
-Vdc/4	S4,S5,S6,S7	S2’,S3’,S4’,S5’	S1,S2,S3,S8	S1’,S6’,S7’,S8’
-Vdc/2	S5,S6,S7,S7	S1’,S2’,S3’,S4’	S1,S2,S3,S4	S5’,S6’,S7’,S8’

Table.1. Switching combinations for a five-level MMI

Their corresponding switching states and voltage output levels for five level MMI as listed in table 1. State 1 of switch means the switch is ON, and 0 means the switch is OFF, where Va is the pole voltage measured between one of the Phase of MMI and neutral. In order to maintain the equal voltage stress on the switching devices, the voltage across all the sub-module capacitors of the MMIs must be retained at Vdc/2. The number of SMs that are connected in the upper and lower arm decides the voltage level of the inverter.

3. Modeling of Modular Multilevel Inverter

3.1 Mathematical Modelling of Modular Multilevel Inverter

The mathematical modelling of MMI is obtained by applying Kirchhoff’s voltage law to the MMI circuit (figure 2), the supply voltage V_{dc}, can be expressed by,

$$V_{dc} = \sum_{h=1}^{2n} V_{hu} + L_{arm} \frac{d}{dt}\left(i_{pu} + i_{nu}\right) \qquad (1)$$

Where, $V_{hu}$is the output voltage of the h[th] sub-module in phase u, i_{pu} is the positive arm current i.e. upper arm current, $i_{nu}$is the negative arm current i.e. the lower arm current, n is the number of sub-modules in each arm. Hence all sub-module voltages are added.The phase-u currenti_u, and the upper and lower arm currents of phase u expressed by Eq.(2.a)-(2.c) respectively. i_{circ}represents the circulating currents in phase u.

$$i_u = i_{pu} - i_{nu} \qquad (2.a)$$

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$$i_{pu} = i_{Zu} + \frac{i_j}{2} \qquad (2.b)$$

$$i_{nu} = i_{Zu} - \frac{i_j}{2} \qquad (2.c)$$

From Eq. 2 the circulating current i_{circ}can be expressed as,

$$i_{circ} = \frac{i_{pu}+i_{nu}}{2} \qquad (3)$$

The circulating current has a frequency twice the fundamental frequency and is sequence form. The circulating current causes a higher RMS value of the arm current, thus increasing the losses in the converter. It also influences the distribution of both voltage and losses [14].The second harmonic behavior of circulating current is given as,

$$i_{circ} = I_{2f} \sin(2\omega_0 t + \varphi_0) \qquad (4)$$

Where I_{2f} , is the amplitude of double line frequency circulating current, ω_0 is the fundamental frequency and φ_0 is the initial phase angle. Based on the equations (2) and (4) the arm current has three main frequency components: Zero frequency DC-current, fundamental frequency AC-current and the circulating current. [15]

3.2 Multi carrier PWM Techniques for Modular Multilevel Inverter

PWM techniques play an enthusiastic role to get the preferred output in multilevel inverters. Numerous modulation techniques have been proposed and bring together in the literature such as: selective harmonic elimination PWM [16], space vector PWM, Sinusoidal PWM, Third Harmonic Injection PWM [17]. The main challenge associated with the SHE-PWM and SVPWM techniques are that they are convoluted because the solution of a large number of equations has to be solved to get optimal switching angles.

The Sinusoidal PWM and Third Harmonic Injection PWM techniques are simple among the modulation techniques. The basic principle of SPWM switching scheme is each time the triangular carrier wave crosses the sinusoidal reference signal, the switch will change position. This can be expanded to multi carrier PWM techniques. In MCPWM technique the modulating signal (sine waveform) is compared to the multicarrier waveforms to produce the gating pulses to the power electronic devices for any output level inverters [18]. MCPWM can be categorized as level shifted PWM (LS-PWM) and Phase shifted PWM (PSPWM) techniques. Typically the LS-PWM is preferred since it improves the quality of the output waveform. The level shifted PWM (LS-PWM) techniques are Phase Disposition (PD), Phase Opposition Disposition (POD) and Alternative Phase Opposition Disposition (APOD) [19].

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In LSPWM technique, sine wave is taken as reference waveform of amplitude (Ar) and frequency (fr), and it is continuously compared with carrier waveform of amplitude (Ac) and frequency (fc). If the modulating signal is greater than the triangular wave, the IGBT is turned on, otherwise it is turned off. For N number of output levels, N-1 carrier wave is needed with identical frequency fc and identical amplitude Ac. The amplitude and frequency of reference waveform is Ar, &fr respectively and it is zero centered in the middle of the carrier set. Each carrier signals are incessantly compared with modulating signal waveform and if the reference is larger than a carrier signal, then thedevice corresponding to that carrier is switched on, otherwise switched off [20], [21].

In general the amplitude modulation index (ma) for level shifted PWM technique is demarcated as the ratio of amplitude of the reference sine waveform (Ar) to the amplitude of the carrier waveform (Ac) and it is given in equation (5).

$$m_a = \frac{A_r}{A_c} \qquad (5)$$

Similarly the frequency modulation index (mf) for level shifted and phase shifted PWM techniques is defined as the ratio of frequency of the carrier wave (f_c) to the frequency of the reference sine wave (f_r) and it is given in equation (6).

$$m_f = \frac{f_c}{f_r} \qquad (6)$$

Level Shifted PWM (LS-PWM)

In LS-PWM methods all the carrier signals are of identical amplitude and frequency. They are categorized fundamentally based on the location of various carrier signals. For generation of *n* level, n-1 carrier signals are necessary. The LS-PWM method is used in the controlling of inverters where power balancing was not required. The LS-PWM technique can be classified as PDPWM, PODPWM, and APODPWM depends upon the phase disposition of the carrier waveforms.

Phase Disposition PWM (PDPWM)

The PDPWM is the generally used scheme for conventional multilevel inverters and Modular Multilevel inverters because it provides load voltage and current with lower harmonic distortion. In the PDPWM method all the carriers above and below zero reference line are in phase.

Phase opposition disposition PWM(PODPWM)

In PODPWM technique all the carrier waves have the identical frequency and the adjustable amplitude (different or unequal amplitudes). In this technique two kinds of carrier wave are used such as positive carrier wave (above the zero value) and negative carrier wave

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(below the zero value).All the carrier waveformsbelowthe zero valuereference are shifted by180°with respect to the positive carrier waveforms.

Alternate phase opposition disposition PWM(APODPWM)

In APODPWM method all the carriers have the same frequency and the adjustable amplitude (different or unequal amplitudes). All the carriers are alternatively displaced 180° between them.

Phase Shift PWM (PSPWM)

A phase shifted carrier PWM for multi-level inverter is used to generate the stepped multi-level output voltage waveform with minimum harmonics. A MMI with N level requires N-1 triangular carrier waveforms. In PSPWM technique, all the triangular carriers have equal frequency and equal peak to peak amplitude, but there is 360°/N phase shift between two adjacent carrier waveforms. The switching signals for five level MMI with Phase Disposition PWM technique is shown in figure 2.

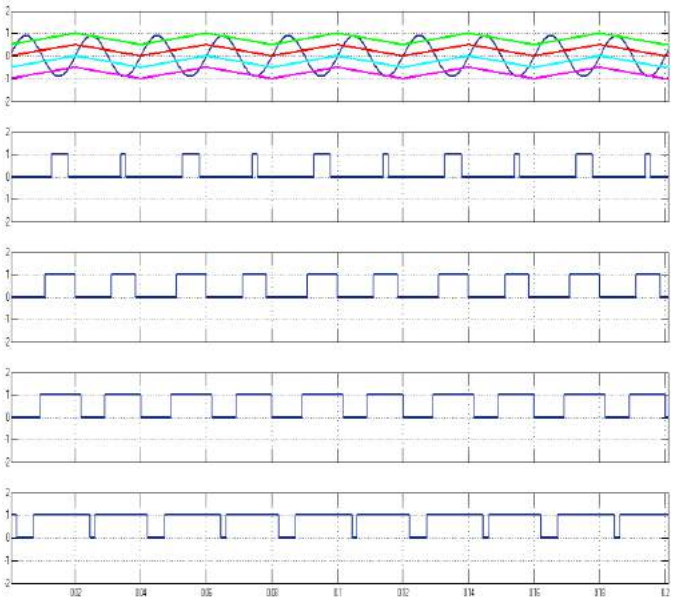

Fig.2. Firing Pulses for Five Level MMI with PDPWM Techniques

3.3 Nearest Level Modulation Method

An NLM technique directly calculates the switching states and duty cycles for each phase of the inverter without requiring carrier signal. NLM brings more flexibility andeasy digital implementation, even though the number of levels of the inverters is large [22]. In NLM method the high number of available voltage levels is achieved by approximating the reference to the closest available voltage level resulting in natural fundamental switching

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frequency with reduced switching losses [23]. MMI with large number of sub-modules can be easily implemented in closed loop and high bandwidth application with NLM.

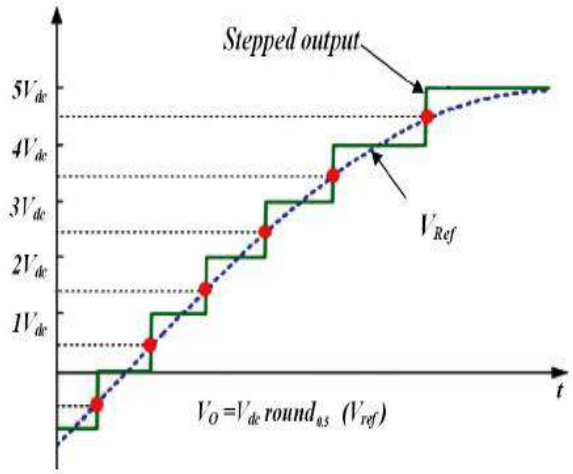

Fig.3. Principal of nearest level Selection for Eleven Level MMI

In [24] conventional NLM was used to generate steps with base concept of round off technique for eleven-level MMI as shown in figure 3. The level control technique is simpler and normalized value is evaluated using round-off method (i.e., $f_{round}\{x\}$ is the integer close to x). The half-integer type is always rounded to even numbers, and the maximum error (DC loss) is 0.5 Vdc. The largest possible error is then limited by half of the DC source voltage (Vdc/2). The firing angle calculation for the five-level MMI is similar to the eleven level for each switch should be such that, on overlapping a sine waveform and the multilevel output waveform of equal peak voltage, the overlapped sinusoidal waveform cuts through the rising edge of the multilevel output at exactly half of its magnitude. The switching angles are given by,

$$\theta_i = \sin^{-1}\left(2i - 1/n - 1\right), \text{Where i} = 1,2, \ldots . \text{n} - 1/2 \qquad (7)$$

4. Simulation Results And Discussion

In this study, three phase MMI was considered and its performance was analyzed for different levels with carrier based PWM techniques and Nearest Level Modulation in terms of Total harmonic distortion of phase voltage, line voltage and line current in MATLAB/ Simulink. Parameters chosen for simulation are given in the Table 2. In this study, three, five, seven, nine and eleven levels are considered to find the effectiveness of MMI using carrier based PWM methods. The output phase voltage, Line voltage and output current are presented in detail for seven level MMI alone as shown in Figure 4, 5, 6 and 7for the modulation Index of 0.9.

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Parameter	Value
Number of submodules	n-1
DC Link Voltage	500V
Submodule Capacitance	4.7 µF
Arm Equivalent Resistance	0.04 Ω
Arm Inductance (L)	2.13 mH
Switching frequency	2 kHz
Voltage levels(n)	N+1
R-L Load	R=9.12 Ω,L=21.3 mH

Table .2. System Parameters

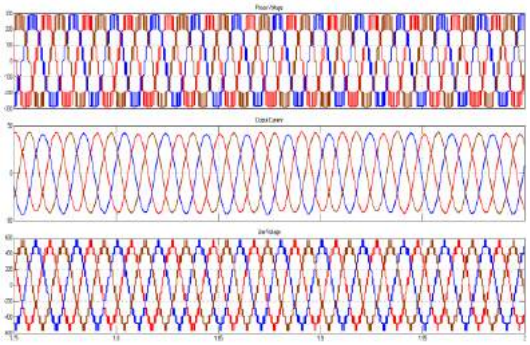

Fig.4. Output Phase voltage, Line voltage and output current of Seven Level MMI

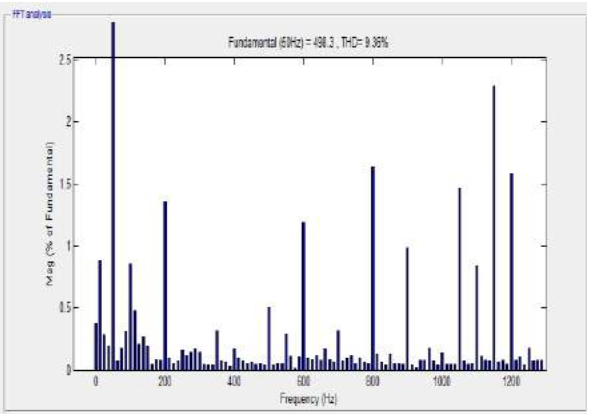

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Fig.5. THD spectra of Line voltage for Seven Level MMI with RL Load

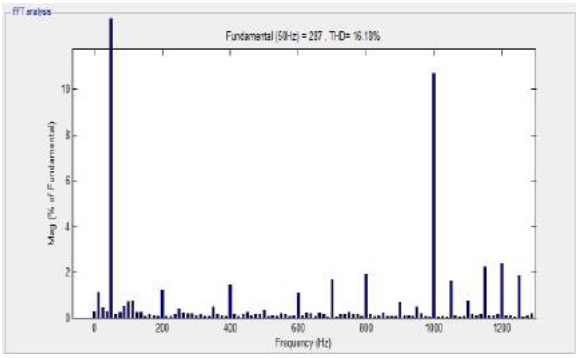

Fig.6. THD spectra of Phase voltage for Seven Level MMI with RL Load

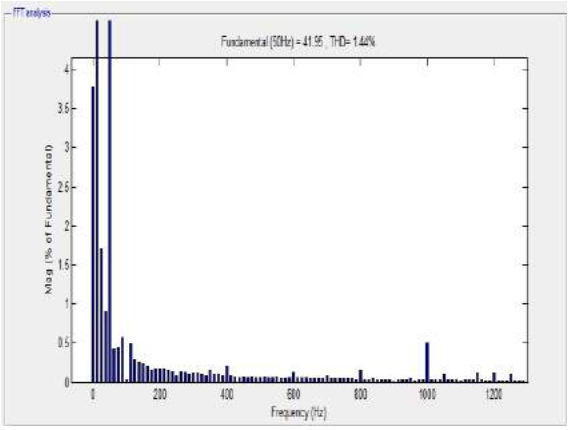

Fig.7. THD spectra of Output Current for Seven Level MMI with RL Load

No. of Level	Total Harmonic Distortion (%)								
	Phase Voltage			Line Voltage			Line current		
	Phase Disposition PWM	*Phase opposition disposition PWM*	*Phase Shift PWM*	*Phase Disposition PWM*	*Phase opposition disposition PWM*	*Phase Shift PWM*	*Phase Disposition PWM*	*Phase opposition disposition PWM*	*Phase Shift PWM*
3	**34.35**	64.89	63.60	**42.22**	54.05	54.95	**2.82**	2.92	5.31
5	**31.51**	31.53	30.88	**16.21**	27.81	26.98	**1.84**	2.08	2.18
7	**16.18**	17.82	16.52	**9.36**	14.27	13.20	**1.44**	2.16	2.63
9	**13.82**	13.98	16.05	**9.70**	12.42	13.14	**1.38**	2.09	2.54
11	**12.63**	13.79	11.21	**8.47**	12.18	9.66	**1.31**	2.04	1.43

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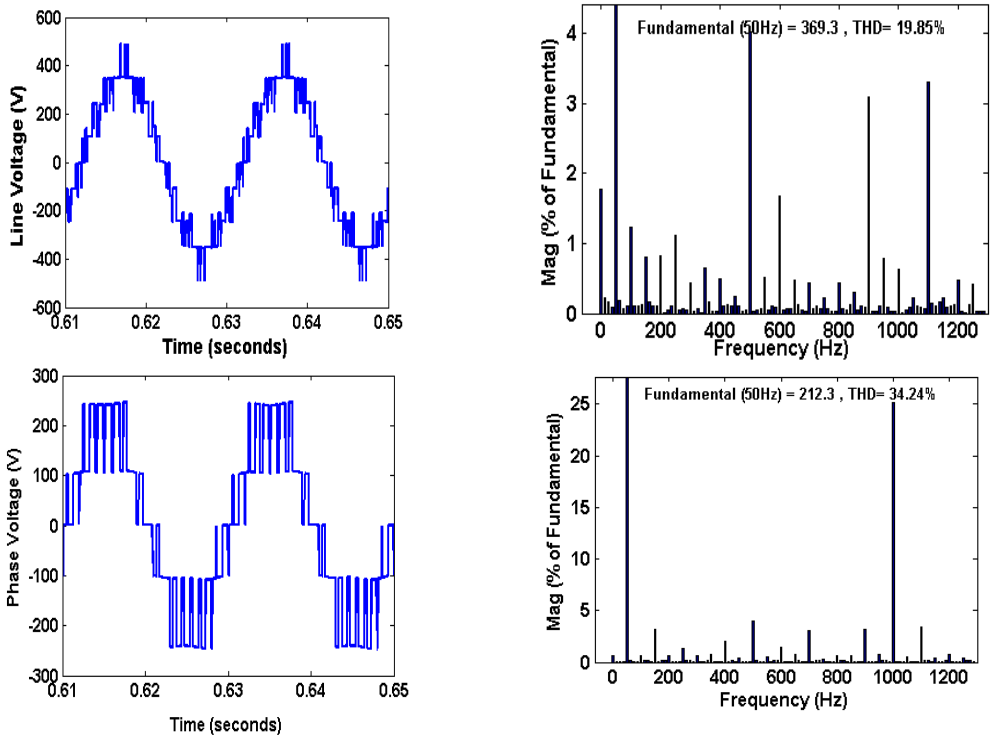
Table.3. Comparison of Various Levels of Multilevel Inverter with Multi Carrier Based PWM Methods

Table 3 summarizes the THD value of voltage and current waveform measured for various levels of MLI considering carrier based PWM schemes. From the table it is found that the THD of the phase voltage, line voltage and output current for the three level MMI with PDPWM are 34.35, 42.22 & 2.82 respectively. It is evident that the Phase Disposition PWM method results in the lowest THD and better RMS value of voltage and current when compared to other carrier based switching schemes like PODPWM and PSPWM. Further, near sinusoidal load current waveform isachieved with PDPWM. From the analysis it is found that as the number of level increases, the THD in the current and voltage waveform reduces i.e. the waveform is closer to sinusoidal. Among the various carrier based PWM techniques, PDPWM technique will be a reasonable choice for high level MMI in terms of improved efficiency. However, high switching frequency is required for Multi-Carrier based PWM techniques which lead to high switching losses. Moreover, the circuit design seems to be complicated and so the real time implementation of Modular MLI using carrier based PWM is a challenging one.



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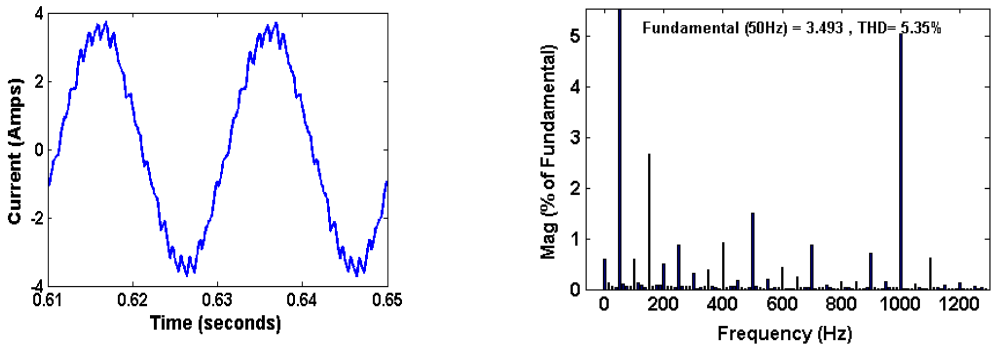
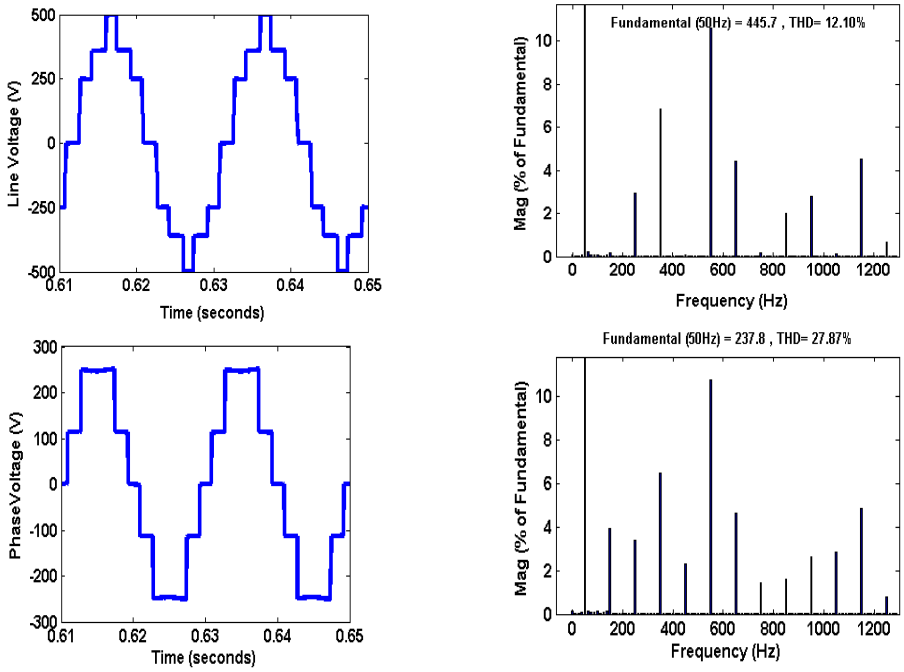


Fig.8. (a) Output Phase voltage (b) Line Voltage and (c) Output current waveform with its harmonic spectrum of the five level MMI with PDPWM

Figure 8 and 9 present the output waveforms and FFT spectrum of Total harmonic distortion of phase voltage, line voltage and load current using PDPWM and NLM. In this modulation technique, the switching frequency of all Sub-modules in each arm is approximately N times of the carrier frequency, Nfcr. With high switching frequency,switching losses are the major issue and so the PDPWM Technique is suitable only for low level MMI.



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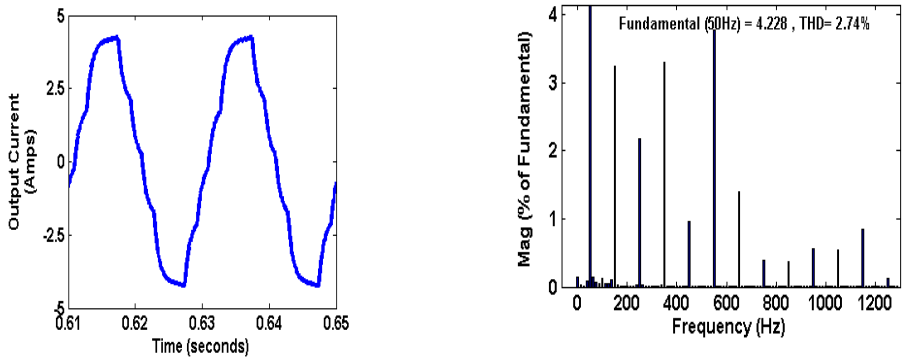

Fig.9. (a) Output Phase voltage (b) Line Voltage and (c) Output current waveform with its harmonic spectrum of the five level MMI with NLM

From figure 9, it is evident that NLM out performs than PDPWM in terms of reduced THD values in voltage and current waveforms and better power quality.

Method	***THD %***		
	Line Voltage	***Phase Voltage***	***Output Current***
NLM	**12.10**	**27.87**	**2.74**
PDPWM	19.85	34.24	5.35

Table.4.Harmonic Performance Comparison of 5-level MMI using PDPWM and NLM

The THD of the output voltage and current for NLM and PDPWM technique are summarized in Table 4 for modulation index of 0.85. It is found that the line voltage magnitude is enhanced to 445.7V with NLM. Further near sinusoidal output waveform with reduecd THD of 12.1 % in line voltage can be achieved with NLM technique ithout using any filter circuits. By applying NLM to Five-level MMI, the percentage reduction in THD in the line voltage, phase voltage and line current are found to be 39.04, 18.6 and 48.48 respectively. From the above analysis it is evident that NLM technique is the most appropriate choice for high level Modular Inverters.

Conclusion

In this paper, a three phase MMI based on half bridge arrangement was considered and simulated inMATLAB/Simulink software using carrier based PWM techniques like PDPWM, PODPWM, PSPWM and Nearest Level Modulation technique. The following conclusions are arrived based on the simulation results.

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(a) By using the PDPWM method, the line voltage and the line current with reduced THD valuesare obtained for the modulation index of 0.9. Further, the RMS value of the line voltage is also better. Thus, the PDPWM method is more suitable for the MMI among all other Multi-carries based techniques.

(b) It has been observed from the analysis that the THD value reduces with increased levels. However, complex control circuitry is required which in turn increases theoverall cost. Therefore it could be concluded that the medium level MMI is best suited for low and medium voltage applications.

(c) Nearest Level Modulation method offers best performance in terms of reduced harmonics with reduced switching frequency when compared to all carrier based modulation techniques. Also, fundamental frequency can be used as switching frequency for generating gating pulses and it can be easily extended to higher levels. Thus, the NLM method is more suitable for higher level MMI because of reduced switching losses and improved power quality. Also, high power output can be realized by integrating the sources.

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Article 11

Emerging Trends of Bi Based Nano Materials in Biomedical Application

Kamala Mitra

Abstract

During the past few years, various nanomaterials have drawn great attention and are well explored in the field of biomedical application. Researchers in nanoscience are continually on the lookout for effective particles that might lead to the creation of novel medications to aid in the early detection and treatment of severe illnesses. For this goal, a variety of nanomaterials are being investigated among which Bismuth nanoparticles (BiNPs) has proved to become interesting in various dimension due to some exceptional properties like large surface area, low toxicity. Within this review, the latest data of biomedical application of Bi-based nanomaterial have been focused on including the aspects related to antimicrobial activity, drug delivery, bioimaging, and biosensors.

Keywords nanoscience, nanomaterials, binanoparticle, biomedical applications

1. Introduction

Nanoscience and nanotechnology is a large, multidisciplinary scientific field that has sparked attention throughout the world in recent years. Nanostructured materials are unique as their optical and electronic properties are size-dependent that arises from the quantum size effect and presence of a large number of surface atoms. Recently, researchers are interested in developing new ways for creating innovative nanomaterials with varied compositions, morphologies, and hybrids, which are critical for reaching high-performance applications. There are plenty of nanomaterials among which Bi-based nanomaterials have attracted great attention with diverse compositions being used for biological purposes due to low toxicity and environment-friendly characteristics.(Yang et al.,2015) Besides Bi's inexpensive price and abundance make it appealing for large-scale applications.(Go´mez-Vela´zquezetal.,2018) The properties of semimetal Bulk Bi which is responsible for making Bi NPs with various shapes and compositions are long Fermi wavelengths, high magnetoresistance, and high diamagnetism.(Smith et al.,1964) In comparison to other metals like silver, Bi is regarded as the least toxic and physiologically non-reactive heavy metal, making it more suited for use in-vivo. One of the main objectives of biomedical researchers is to find effective particles which might result in the establishment of novel drugs. To give a new dimension to this investigation Bismuth oxyhalides (BiOx) and bismuth chalcogenides have been extensively studied for medicinal applications. Bi_2O_3, Bi_2S_3, Bi_2Se_3, and Bi_2Te_3 are the most frequent Bi chalcogenides, which belong to group VI of Bi compounds. The intrinsic electrical and optical characteristics of Bi chalcogenide nanostructures make them appropriate for a vast range of biomedical applications(Fowler et

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al.,2007) whereas morphology and crystal structure plays a great role in the modification of these properties. (Nietal.,2017) Another class of Bi compounds is Bi oxyhalides (BiOX, where X is Cl, Br, or I) that pertain to the ternary oxide semiconductor materials V-VI-VII. So far the different morphologies of Bi nanostructure have been synthesised are nanospheres, nanocubes, nanowires, nanorods, nanotubes, nanoplates, nanosheets, and thin films. This review demonstrates the synthesis and the progress of Bi-based nanomaterials in biological applications such as drug delivery, antimicrobial activity, bioimaging, cancer therapy, and biosensing with different compositions, and structures.

2. Synthetic Procedures of Bi nanoparticles

By varying the concentration of the source of Bi, the hydrothermal method offers energy-saving and cost-effective benefits in synthesising bismuth nanoparticles BiNPs with controlled particle size, shape, and degree of crystallinity.(Zulkiflietal.,2018) A polymer-assisted hydrothermal technique was used to synthesise $BiFeO_3$.This synthesis incorporates the usage of poly(vinyl alcohol) (PVA) to prevent the crystals from expanding to micrometer size. (Wanget al.,2008) Surfactants have the capacity to direct morphology by controlling crystal formation to create nanostructures with a number of different morphologies. Based on this Bi_2Te_3 nanostructures was prepared using three different surfactants like ethylene glycol (EG), poly(vinyl pyrrolidone) (PVP), and ethylenediaminetetraacetic acid (EDTA) which resulted in the formation of nanospheres, nanoplates, and nanoflakes, respectively.(Dharmaiahetal.,2017) Due to low interfacial tension, ionic liquids are better used for the production of small particles than traditional organic solvents. The synthesis of bismuth di-n-octyl-dithiophosphate ($Bi[S_2P(OC_8H_{17})_2]_3$) was demonstrated in tetrafluoroborate salt of 1-Hexadecyl-3-methylimidazolium-bromide $C_{16}MIMBF_4$ as an ionic liquid solvent. (Wangetal.,2010) The sol-gel method is mostly used for the preparation of thin-film and coatings which helps to fabricate sub-micron metal oxides. With the addition of chelating agents like acetic acid, tartaric acid, etc. the purity and morphology of the final products were under control. A tartaric acid-assisted sol-gel process was used to create pure $BiFeO_3$ of polyhedral shape.(Wangetal.,2010) A microemulsion technique for capping Bi_2Te_2 NPs with thioglycolic acid to avoid agglomeration as well as surface oxidation was developed.(Purkayasthaetal.,2006) Synthesis of metallic BiNP was performed by the reduction of $Bi(NO_3)_3.5H_2O$ with help of strong reducing agent $NaBH_4$ in a solution of glycine and dextran which acted as a surfactant.(Brownetal.,2012) In the microwave irradiation procedure, the ionic liquid 1-n-butyl-3-methylimidazolium tetrafluoroborate ($[BMIM]BF_4$) was utilised as a solvent, to generate pure hexagonal Bi_2Se_3 nanosheets.(Jiangetal.,2006) After cell exposure to diverse kinds of BiNPs, thorough research on reactive oxygen species (ROS) production, the toxicity of mitochondria, damage of cell membrane, and DNA fragmentation, as the key mechanisms of NP-induced cellular damage, where required. The cytotoxicity of the NP is highly affected by the particle size, charge and ,modification of the surface. The main toxicity of BiNPs are the leaching of free

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Bi^{+3} ions in the bloodstream. This was prohibited by designing biologically stable PVP coated Bi_2S_3 NPs which were highly dispersible in an aqueous medium.(Esquivel-Gaonetal.,2015) The biomimetic coating of NPs with red blood cell membranes (RBCs) has been attracting considerable interest for active inhibition of immunological assault and the prolongation of blood circulation.(Xiaetal.,2019)

2.1.Bio-security of Bi nanoparticles

In order to cultivate the biological response and safety of oral administration of BiNPs Bi_2S_3@SiO_2 nanorods were synthesized.(Zhengetal.,2015) *Caenorhabditis elegans (C. elegans)* worm was chosen for the model organism in association with lipofuscin a biomarker. From the observation of the study of imaging potential of Computed tomography (CT) and photoacoustic tomography (PAT) of nano rod, it was suggested that BiNP can function as a multi-modal contrast agent for direct observation of gastrointestinal tract with less toxicity. Because of the genome's unique closeness to the genome of humans, zebrafish is a viable model of animal for drug formulation research. Bi-asparagine coordination polymer toxicity (BACP-2) was tested using zebrafish embryos.(Heetal.,2013) The investigation illustrated that there were no negative consequences regarding the NP. These searching provide light on potential problems and the requirement for thorough toxicity testing of different forms of BiNPs before they are used in biomedicine. The nephritic (urine) and hepatic (bile to feces) pathways are the two primary mechanisms for NP elimination. The biosafety of nanoparticles depends upon how quickly they are removed from the body after being administered.(Soo Choietal.,2007) As $Cu_3BiS_3$and $(BiO)_2CO_3$ nanotubes being easily degraded by acid was efficient in clearance by the kidney. The ultrasmall size of Bi–Bi_2S_3 heterostructure NPs also led to its removal by urine. The size of the nanoparticle plays a great role in this excretion.(Dongetal.,2019)

2.2.Drug delivery approach

The fast advancement of medicine has prompted the progress of innovative drug delivery methods that allow drugs to be released efficiently and securely in the human body to treat numerous ailments.(Figueiredoetal.,2016) New BiNPs have been created to boost their capability for transporting drug molecules.Great stability and porosity of Poly(ethylene glycol) mediated Bi_2S_3 nanourchins was successful for drug loading like Doxorubicin(DOX). The function of Poly(ethylene glycol) was observed to increase the blood circulation of nano particles.According to the report it was found that DOX was encapsulated and protected with mesoporous silica coating Bi_2S_3 (Bi_2S_3@MSN) NPs in addition DOX and NP were combinedly loaded in nano hydrogel prepared from carboxymethyl cellulose(CMC) and Bi citrate (Bi@CMC nano hydrogel).(Xuanetal.,2019) The graphene nanosheets decorated with Bi_2S_3 (PVP–rGO/Bi_2S_3) nanocomposite had a capacity of drug loading ~ 500 percent for DOX. This was significantly higher than previously reported loading values for chalcogenides in nanoscale. The huge area of the

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surface of rGO and the secure p–p stacking interaction among the medication and rGO were primarily responsible for this capacity. (Douetal.,2016)

2.3.Imaging contrast agent

Due to the ability for deep-tissue imaging with great resolution, Computed tomography imaging(CT) is one of the most popular used imaging techniques. So far various elements has been implemented for Contrast agent in CT imaging but Bi has proved its high efficiency because to its low cost and high biosecurity. Bi_2S_3 nanorods coated with Tween 20 was applied in tumour bearing mice in order to obtain CT imaging and the excellent result was obtained about the contrast signals. Surface coatation promoted a strong effect of EPR and a long residence period of the particle in the tumour for CT imaging.(Liuetal.,2015) Poly(ethylene glycol)(PEG) mediated metallic Bi nanocrystals (Bi–PEG NCs) were observed to have the potential of high-performance CT imaging diagnostic agent.(Lietal.,2017) As the signals were much higher images with great brightness and resolution were obtained comparative to any other agent. The Bi–PEG nanocrystals had a high coefficient of X-ray absorption of ~60.3 HU mL mg^{-1}, 3.7 times greater than commercially supplied iopromide (~16.4 HU mL mg^{-1}). The size of the NPs plays an important effect in the basis of EPR accumulation(Bietal.,2018) in a tumours. Keeping this in mind extremely small Bi–PEG nanodots and Bi nanodots with PVP protection with sizes of 4nm and 2.7 nm respectively was developed .(Leietal.,2017) Because of their increased accumulation in cancer tissue, they operate better for tumour imaging at extremely low doses. The use of nanostructures in infrared thermography(IRT) imaging has also created a lot of interest. The process of converting infrared energy released by an object at a certain temperature and displaying the end outcome as an infrared picture is known as infrared thermography. As a good imaging contrast for IRT Bi nanodots with PVP-protection were utilised for this purpose.(Zhangetal.,2013) Bismuth selenide (Bi_2Se_3) nanoparticle coated with Polydopamine (PDA)/ human serum albumin (HSA)/doxorubicin(DOX) (Bi_2Se_3@PD/ DOX/HSA) NPs were also observed to be a good IRT imaging contrast agent.(Lietal.,2016) For expanding the imaging potential, Bi-nanocomposites have also been studied for Magnetic resonance imaging(MRI) and ultrasound.MnSe@Bi_2Se_3 core-shell nanostructure was applied as a contrast agent for studying MRI. MnSe core acted as contrasts for MRI whereas Bi_2Se_3 shell was responsible for strong absorption of X-rays and NIR light both.(Songetal.,2015)

2.4.Anti cancer treatment

Surgery, radiation therapy, chemotherapy, hormone therapy, and biological therapy are all renowned options for cancer treatment. Depending on the kind and site of cancer, progress of the disease, the patient's age and, general health, and other considerations, one therapy or a mix of techniques may be utilised. Tremendous research is going on in this sector to come up with new ideas of anti-cancer agents. It was observed that Bi NPs have the

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potential to act efficiently in this treatment. Photothermal therapy (PTT) is a cancer treatment that uses heat to kill cancer cells by exposing tumour tissues to near-infrared (NIR) light. (Liuetal.,2015) NIR absorbents are utilised to make heat generation more efficient. Some BiNPs have become good cancer PTT nano agents, because of their great NIR absorbance,(Zhouetal.,2018) a high rate of photothermal conversion, and good biosafety.(Zhangetal.,2017) Flower-like Bi_2S_3 NPs, Bi_2S_3–Au heterojunction Nanorods, $Bi_2S_3/Cu_2S/Cu_3BiS_3$ composites,(Yuetal.,2018) PVP–Bi_2Se_3 nanosheets having the efficiency of photothermal conversion as 64.3%, 51.06%, 43.8%, and 34.6% respectively were observed to act as highly desirable agent(Chengetal.,2018) for abolishing cancer tissues *in vivo*.(Xieetal.,2016) In radiation therapy to destroy cancer cells and reduce tumours, radiation of high-energy such as x-rays, gamma rays, protons, and other sources are used. The effective property of photoelectric absorption of BiNPs was reflected on Bi_2S_3 encapsulated Poly(lactic-co-glycolic acid)(PLGA) nanocapsules (Bi_2S_3@PLGA).(Huangetal.,2014) After being exposed to gamma-ray irradiation Bi_2S_3 acted as a sensitizer to radiation for the demise of PC3 prostate cells. Another aspect of tissues from cancer that may shield it against radiation therapy damage is its concentration of antioxidants. The conversion of intracellular H_2O_2 to the highly poisonous •OH increases the production of reactive oxygen species, which causes malignant cells to die. To achieve this bismuth heteropolytungstate (BiP_5W_{30}) in combination with reduced graphene oxide (rGO) and PVP modified surface was effectively applied to the human cervix adenocarcinoma cell line (HeLa cells).(Zhouetal.,2019)

2.5.Antimicrobial activity

Inhibitory effect of colloidal Bidimercaptopropanol (BisBAL) on development of biofilms of *Pseudomonas aeruginosa (P. aeruginosa)* (Badireddyetal.,2014) and generation of *S. mutans and S. gordonii* was reported. (Badireddyetal.,2013) Mineral trioxide modified BisBAL NPs were incorporated to reduce the chance of infection. The application of the NP demonstrated that the increase of *Enterococcus faecalis(E. faecalis), E. coli,* and *C. albicans* might conceivably be inhibited.(Hernandez-Delgadilloetal.,2017) To investigate the property of antibacterial mechanism of BisBAL NPs it was assumed that dissolution of NP leads to the release of Bi ion which reacted with the respiratory enzymes containing sulfhydryl groups after penetrating the cell wall. This was the cause of the breakdown of the cell wall which deactivates the bacteria.(Badireddyetal.,2013) Anti-*P. aeruginosa* antibodies were used to develop surface-modified BiNPs which exhibited an excellent effect for the healing of wounds infected by bacteria.(Luoetal.,2013) Polyethyleneimine (PEI) coated Bi_2Se_3 nanoplates destroyed *S. aureus* and *E. coli* bacteria with the efficacy of nearly 99% and 97% respectively. The concentration of NPs only 80 parts per million (ppm) was effective for this eradication.(Gorleetal.,2018) Bi_2Se_3 nanodiscs were used to eradicate Gram-positive *S. aureus* bacteria by scavenging intracellular reactive oxygen species which lead to a new dimension for the treatment of infected wounds.(Ouyangetal.,2019)

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2.6. Biosensor

Biosensors are diagnostic devices that incorporate a biological sensing element, or biorecognition element, that is responsible for specificity, and a physical transformer that translates the recognition phenomena into a measured signal. A component that detects the analyte and produces a signal, a signal transducer, and a reading device makes up a biosensor.

Generally, nanomaterial-based biosensors have advantages over the conventional ones for their quick reaction, great sensitivity, wide surface area, and mobility.

2.7.Bismuthoxybromide based Biosensor

In order to detect insulin, a bismuth oxybromide/silver sulphide (BiOBr/Ag_2S) combination with strong visible-light photoelectrochemical(PEC) activity was developed. As the separation of photogenerated hole/electron is inefficient under visible light because of the large band-gap of BiOBr, hence Ag_2S NPs are utilised to facilitate the transition of the electron under visible light.(Fanetal.,2017) Based on a PEC immunosensor a unique BiOBr/Bi_2S_3 composite was used to detect squamous cell carcinoma antigens (SCCAs).(Fanetal.,2019) Bi_2S_3 has a high valence band (VB) and conduction band (CB), which speeds up electron transport when exposed to visible light and increases the conductivities of photoelectrochemical biosensors. Flower-like carbon nitride/bismuth oxyhalide (CN/BiOBr) composites was used for detection of broad-spectrum antibiotic tetracycline .(Yanetal.,2018) Metallic Bi self-doping BiOBr (Bi/BiOBr) composites were applied for the detection of Ciprofloxacin.(Yanetal.,2018) The pervasiveness of these materials and their long-term usage has had a significant negative impact on the environment and human health which may be eliminated by these biosensors.

2.8.Bismuthselenide based Biosensor

Au-encapsulated Bi_2Se_3 nanoparticles were synthesised and eight-silver-ion mediated double-stranded DNA (mDNA) was immobilized on it to produce (Bi_2Se_3@Au-mDNA) for detection of H_2O_2 released from a breast cancer cell.(Mohammadniaeietal.,2018) Bismuth selenide nanoparticles acted as electrochemical sensing enhancers. The manufactured biosensor represents roughly ten times more Ag^+ redox current than electrodes made withoutBi2Se3@Au, according to electrochemical measurements.(Zhuetal.,2015) A rod-like bismuth sulphide nano and polyaniline (PANI) nanocomposite film modified ionic liquid-carbon paste electrode (IL-CPE) has been created as a highly sensitive impedimetric DNA biosensor.(Shahbazietal.,2020) The electrode was ideal for DNA immobilisation and hybridization detection due to its high surface area, superior electric conductivity, and strong biocompatibility.

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Conclusion

The review has discussed the excellent uses of BiNPs in a biomedical applications which is encouraging as a new entry point for medicinal research. The large abundance and less cost of Bi metal have uplifted the interest for more research in the field of Bi-based nanomaterials. To achieve this success and for the commercialization of nanomedicines, priority should be given to the toxicity measurements with great care. It can be proposed that significant development of BiNP research can be responsible for the emergence of innovative applications in biomedicine in the nearest future and will have a positive influence on human welfare.

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Article 12

Phytoplankton Diversity and Community Composition in the Par River Estuary, West Coast of India

Shefali S. Patel and Susmita Sahoo

Abstract

Phytoplankton are considered as the great worth of ecology because of their several beneficial roles in the aquatic ecosystem. This study described phytoplankton diversity and abundance during January to December 2019, in the site 1 (Downstream) and site 2 (Upstream) of Par River estuary, located in the latitude of 20˚53' N and longitude of 72˚89' E with an altitude of 300 m. Total 87 phytoplankton taxa was list out from Par estuary. Bacillariophyta was dominant with 66.66% composition. Chlorophyta and Euglenophyta had similar species composition as 8.045%. Dinophyta reported 6.90% of composition as like Cyanophyta. Charophyta had a 2.30% composition of all taxa. The least composition was showed by Eustigomatophyta is 1.15%. At downstream and upstream, Naviculaceae and Fragilariaceae families were showed the highest family contribution. The study revealed that the summer period exhibited greater diversity and winter exhibited a greater abundance of phytoplankton. The abundance of phytoplankton differs temporally from wet to dry phase as well as spatially from estuarine to fluvial province. The alpha and beta diversity patterns of phytoplankton communities across the Par river estuary were analyzed to exhibit the diversity level.

Keywords phytoplankton, biodiversity, diversity indices, Par river estuary

1. Introduction

The estuary is a most productive natural inhabitation of the world because of the more nutrients content as well as sediments through the influx of fresh and marine water and is a transitional belt between terra and aqua zones (Badarudeen *et al.,* 1996). To reduce the risks of floods, the upper limits of estuaries in industrialized regions have been artificially moved seawards by dams; thereby the fluvial water zone is reduced to reach the tidal area (Muylaert *et al.,* 1997). Generally, the estuaries furnished naturally stressed and highly varied habitats which are subjected to exposure the anthropogenic distraction (Hu & Cai, 2013). Since estuaries have been called the "Nurseries of the Sea", the proper monitoring of diversity and ecology of Phytoplanktonic flora in an estuary is a thrust area of marine biological research in order to enrich many species of fishes, benthos, and birds that depend on this estuary for food and nesting areas. Hence the conservation of the Estuarine Ecosystem is a must in diversity, ecology, and fisheries point of view. An estuary has less attention in ecological research by limnologists and marine biologists because of the intermediate environment between the sea and river (Odum, 1988). The patterns of the phytoplankton community in an

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estuarine water body, especially in tropical oligotrophic systems, are still little known when compared to the extensivenumber of studies carried out on estuarine systems in temperate regions (De Jesus Affe *et al.,* 2018). The tides can cause temporal variations in the phytoplankton community composition, structure, and distribution, especially in shallow coastal waters, where generally this punctuation periodicity is more notable (Blauw *et al.,* 2012). A state of the environment report published in 2012, by the Gujarat Ecological Commission reads, "The peculiarities of the somewhat heterogeneous coastal system and the impact of fast-paced development pose environmental challenges that need to be better understood".

Phytoplankton is a very diverse category of polyphyletic elements, comprising photosynthetic microalgae and cyanobacteria (Reynolds 2006). Biodiversity is one of the essential characteristics of ecosystems. Usually, the marine habitat possesses great phytoplankton diversity, which is influenced by the diatomic elements from benthic communities and periphyton. Biodiversity is a multidimensional property of an ecosystem and diversity indices can provide a good sense of interactions in an ecosystem. The uniqueness and commonness of individuals, as well as species in a community, are revealed through the Diversity indices (Stefanidou *et al.,* 2020). According to Boney, 1975 phytoplankton have significant contributions to the coastal ecosystem especially in an estuary and being important primary producers in the marine ecosystem. In several terrains observed the fishery downfall has been embalmed of plankton population reduction (Santhanam *et al.,* 2019). Marine phytoplankton contributes to up to half of global primary production, providing organic matter for the great majority of marine life and being crucial to the global carbon cycle (Falkowski, 2012). In Gujarat, however, there are a few reports on phytoplankton diversity from estuaries, although a number of publications are available on the occurrence of phytoplankton in brackish and marine waters (Saravanakumar *et al.*,2008; Narmada *et al.,* 2015; Jiyalal Ram, 1991; Jiyalal Ram *et al.,* 1989; Bhavsar and Pandya, 2018; George *et al.,* 2012). The present study dealt with the phytoplankton diversity in Par estuary along with the South Gujarat, West coast of India.

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2.Materials and Methods

2.1. Study Area

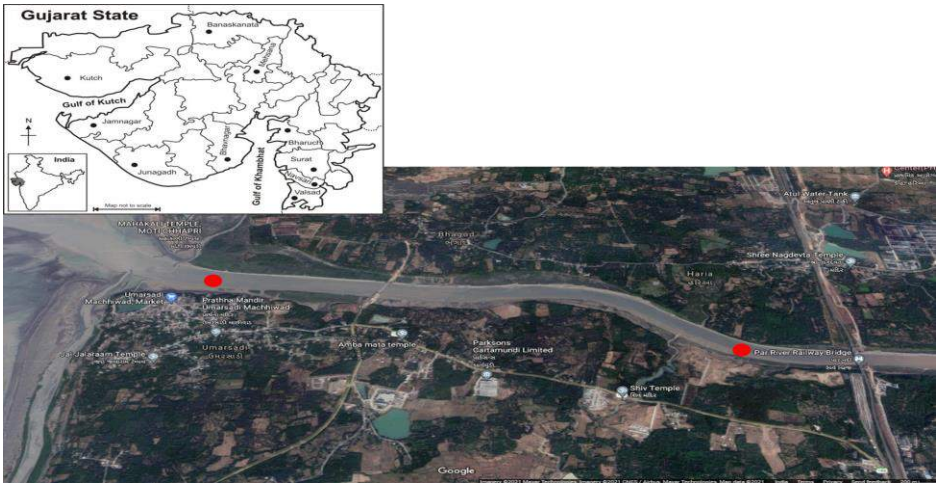

Figure 1: Sampling sites from Par river estuary

Valsad has three coastal major coastal points: Pardi, Umbergaon, and Valsad. Within Valsad, the Umbergaon to Kosamba coastal stretch of 73 km is the most prosperous fishing and fish breeding ground in south Gujarat (As per census data, 2011). The Par river estuary is connected to the Arabian Sea in Umarsadi. A weir dam was formed for the storage of water forAtul industrial complex six km upstream from the estuary's mouth in the 1980s. The complex was to discharge 25000 m^3 per day of wastewater into the estuarine region below the weir dam (Zingde *et al.*, 1979). Geographically, the Par estuary is located in the latitude of 20˚53' N and longitude of 72˚89' E. The selected sites for the present study were (1) Downstream- NearLaxmi Narayan Temple, Umarsadi, and (2) Upstream- Near Atul with a distance of approximately 1-2 km (Figure 1). The sites were selected on the basis of a confluence of fresh fluvial water from upper reaches and coastal water from lower reaches as well as an earlier screening process.

2.2.Sample Collection and Analysis

Phytoplankton samples were collected by towing a plankton net (20 µm mesh) under the water surface of about 1-8 m depth. On each sampling occasion (24 in total), the same procedure was carried out. Subsequently, collected material was concentrated, transferred into 100 ml containers, and preserved with 4% formalin solution (Narmada *et al.,* 2015). The qualitative analyses were carried out by observations on slides, under a light microscope (LABOMED STC-HL) at the magnification of 10x, 40x, and 100x. The taxa were identifiedusing the following references (Joshi *et al.,* 2018; Gopinathan *et al.,* 2007; Hendey, 1957; Bellinger and Sigee, 2010; https://www.algaebase.org/). Quantitative analyses to

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determine phytoplankton abundance (No. L^{-1}) were made according to the aid of standard protocols for plankton counting (Verlecar and Desai, 2004).

3. Results and Discussion

In this case study, a total of 87 phytoplankton taxa was list out from Par estuary.The enlisted species from both sites of this estuary were given in table 1.Among this Bacillariophyta was dominant with 66.66% composition. Chlorophyta and Euglenophyta had similar species composition as 8.045%. Dinophyta reported 6.90% of composition as like Cyanophyta. Charophyta had a 2.30% composition of all taxa. The least compostion was showed byEustigomatophyta is 15%

Phytoplankton	Site 1	Site 2		Site 1	Site 2
Bacillariophyta (Diatoms)			*Pleurosigma elongatum*		*
Asteromphalus heptactis		*	*Proboscia sp.*		*
Asteromphalus flabellatus		*	*Pseudonitzschia australis*	*	
Achnanthidium minutissimum	*	*	*Rhizosolenia hebetata*		*
Achnanthidium pseudolineare	*		*Surirella elegans*	*	*
Achnanthes lanceolata		*	*Surirella sp.*	*	
Achnanthes brevipes		*	*Synedra acus*		*
Amphora ovalis		*	*Synedra ulna*	*	*
Amphora sp.	*	*	*Synedra rumpens*	*	
Amphora marina	*		*Thalassiosira ecentrica*		*
Amphipleura pellucida		*	*Thalassiosira pseudonana*		*
Bacillaria paxillifera	*	*	*Tabellaria fenestrata*	*	*
Biddulphia mobiliensis	*		**Dinophyta (Dinoflagellates)**		
Chaetoceros sp.	*		*Prorocentrum lima*	*	
Cymbella affinis	*	*	*Ceratium fusus*	*	*
Cymbella marina	*		*Polykrikos sp.*	*	*
Cymbella aspera		*	*Peridinium bipes*	*	*
Cymbella tumida	*	*	*Peridinium quinquecorne*	*	
Cymbella lanceolata		*	*Protoperidinium subinerme*	*	*
Diadesmis confervacea	*		**Euglenophyta (Euglenoids)**		
Diatoma vulgaris		*	*Euglena gracilis*		*
Diatoma hyemalis		*	*Euglena proxima*		*

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Diploneis obliqua		*	*Euglenaria clavata*	*	*
Diploneis ovalis	*	*	*Euglenaria caudata*	*	
Fragillaria capucina	*	*	*Phacus elegans*		*
Fragillaria pinnata		*	*Lepocinclis salina*	*	*
Fragillaria sp.		*	*Lepocinclis ovum*		*
Gomphonema louisiananum	*	*	**Chlorophyta (Green algae)**		
Gyrosigma balticum	*	*	*Enteromorpha sp.*		*
Lauderia sp.		*	*Spirogyra porticalis*		*
Melosira monoliformis	*		*Spirogyra tenuissima*		*
Navicula transitans	*	*	*Spirogyra communis*	*	*
Navicula tripunctata	*		*Spirogyra varians*		*
Navicula incerta		*	*Scenedesmus serratus*	*	*
Navicula venerabilis	*		*Cladophora crystalina*		*
Navicula cryptocephala		*	**Cyanophyta (Blue-green algae)**		
Navicula cupsidata	*	*	*Gloeocapsa sp.*	*	*
Navicula salinarum	*	*	*Microcystis aeruginosa*	*	*
Navicula radiosa	*		*Spirulina major*	*	*
Nitzschia dissipata	*	*	*Spirulina princeps*		*
Nitzschia amphibia	*		*Anabaena circinalis*		*
Nitzschia prolongata	*		*Aphanizomenon sp.*		*
Nitzschia scalepelliformis		*	**Eustigomatophyta(Yellow-green)**		
Nitzschia terricola		*	*Nannochloropsis salina*	*	*
Pinnularia conica	*	*	**Charophyta (Green brittleworts)**		
Pinnularia sp.	*		*Klebsormidium flaccidum*		*
Plagiogrammopsis sp.		*	*Cosmarium sp.*	*	
	Total			**49**	**67**

Table 1: Enlisted phytoplankton species from Par river estuary

About 67 species of phytoplankton were recorded from the upstream site and about 49 species were from the downstream site. Bacillariophyta was dominant with 33 species (67.35%) followed by Dinophyta (12.24%), Cyanophyta (6.12%), Euglenophyta (6.12%), Chlorophyta (4.08%), Charophyta (2.04%), and Eustigomatophyta (2.04%) from the downstream site while in upstream site enlisted Bacillariohyta was 42 (62.68%) followed by Chlorophyta (10.44%), Cyanophyta (8.95%), Euglenophyta (8.95%), Dinophyta

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(5.97%), Charophyta (1.49%) and Eustigomatophyta (1.49%) (Figure 2 (A) and (B)). Only one Eustigomatophyta species was found from both sites of this estuary. Charophyta was recorded with two species of phytoplankton. In our study, Bacillariophyta was abundantly present with Dinophyta and Chlorophyta in downstream and upstream sites respectively. The compositions of Chlorophyta and Cyanophyta were predominantly found at the upstream site.

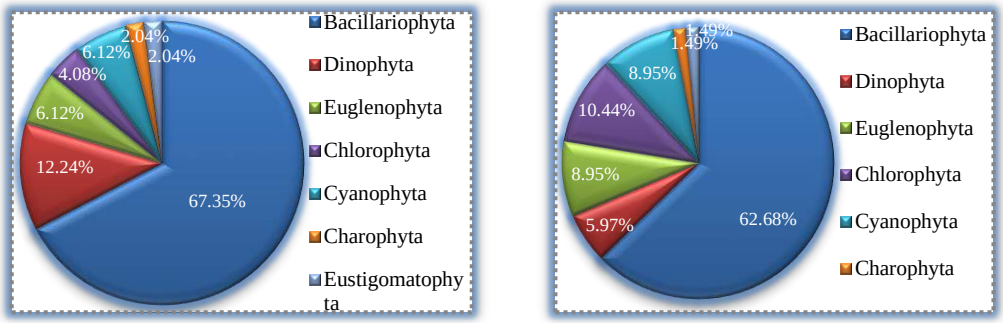

Figure 2: Composition of phytoplankton taxa (A) Downstream; (B) Upstream

At downstream, the Naviculaceae family was showed the highest family contribution, while Cymbellaceae, Fragilariaceae, and Nitzschiaceae families were exhibited the second highest family contribution. The families belong to Cyanophyta and Chlorophyta showed predominant contribution at upstream than downstream. Euglenidae and Phacidae were presented more occurrences at upstream rather than downstream. The families of Bacillariophyta such as Asterolampraceae, Achnanthaceae, Amphipleuraceae, Lauderiaceae, Cymatosiraceae, Probosciaceae, Rhizosoleniaceae, and Thalassiosiraceae were not found from downstream. Charophyta families Klebsormidiaceae and Desmidiaceae were recorded from upstream and downstream respectively. Peridiniaceae family showed maximum composition among all Dinophyta and Microcystaceae familyshowed maximum composition among all Chlorophyta in downstream. The family of Eustigomatophyta called Monodopsidaceae revealed a similar contribution at both sites of the estuary. Nostocaceae and Aphanizomenonaceae were exhibited only from upstream. Cladophoraceae and Ulvaceae were recorded Chlorophyta families also only in upstream. At upstream, the maximum family contribution was exhibited by Naviculaceae and Fragilariaceae families. Bacillariophyta families such as Biddulphiaceae, Chaetocerotaceae, Diadesmidaceae, and Melosiraceae were not found from upstream. The family Cymbellaceae, Euglenidae, and Zygnemataceae showed the second highest family contribution at upstream. The family of Dinophyta Prorocentraceae was not reported from upstream, it was only found in downstream (Figure 3)

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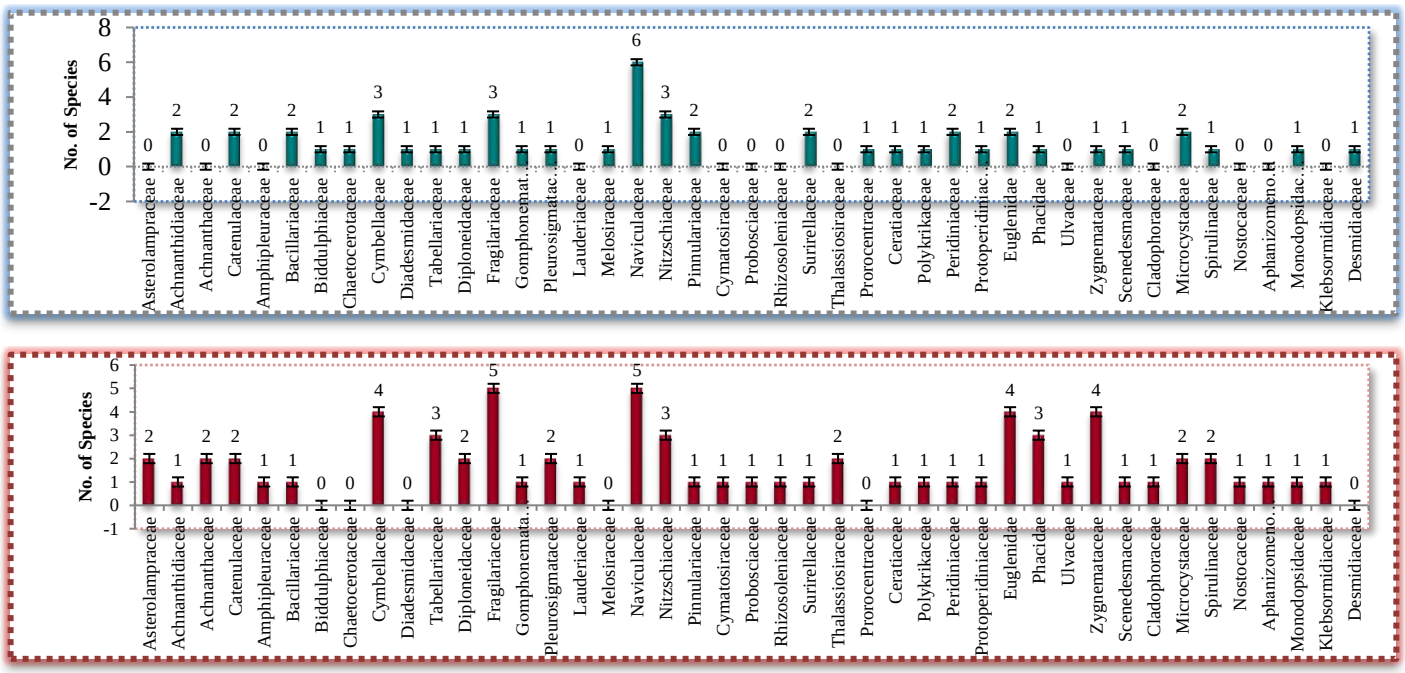

Figure 3: Phytoplankton family contribution

Water management of any coastal water body is clearly understood by phytoplankton diversity patterns. However, the beta diversity of phytoplankton is very little known till today. Beta diversity is a measurement of diverse species composition between two or more habitats. (Stefanidou *et al.,* 2020). In this case study, we revealed the alpha and beta diversity patterns of phytoplankton communities across the Par river estuary. We found significant spatial and temporal differences in alpha diversity of phytoplankton ($p < 0.05$) (Table 3). The Shannon Wiener diversity index was 3.155 and 3.255 atthe downstream and upstream sites respectively. Simpson dominance index was the maximum in downstream (0.4677), while Pielou's Evenness index was the maximum in upstream (0.697).The minimum and maximum Margalef richness index were 1.344 and 1.542 at the respective site upstream and downstream (Figure 4).The beta diversity index called Jaccard's similarity index was 0.80 and Sorensen's similarity index was 0.66 for both habitats (Table 2). The hightest diversity was observed among phytoplankton taxa in summer, which was similar to the funding recorded by Ganjian et al.,(2010).

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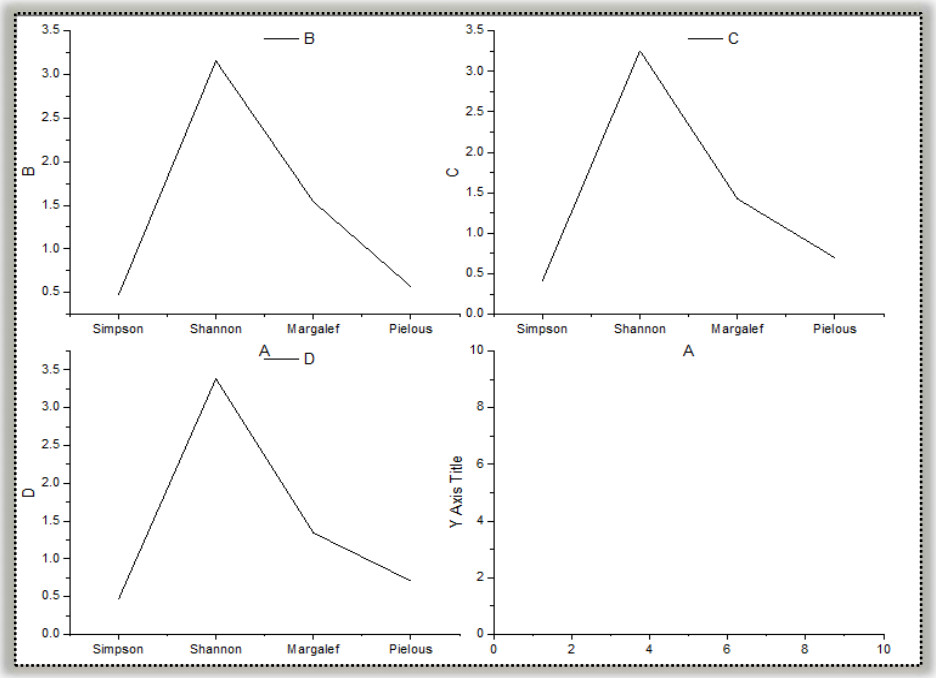

Figure 4: Alpha diversity indices of phytoplankton communities from Par river estuary

Sites	Downstream	Upstream	Total	Beta diversity index
Downstream	1	0.8	0.735	Jaccard's Similarity Index
Upstream		1	0.696	
Total			1	
Downstream	1	0.66	0.581	Sorensen's Similarity Index
Upstream		1	0.535	
Total			1	

Table 2: Beta diversity indices of phytoplankton communities between two habitats

In the present study, the spatial and temporal variations in the phytoplankton abundance were studied using microscopic counts. Bacillariophyceae was present abundantly in both the sites of Par estuary with an average of 836 No/L. Seasonally, summer was recorded with the highest abundant 926 No/L in the upstream and 1304 No/L in downstream. Winter had 761 No/L and 862 No/L in upstream and downstream respectively. Monsoon had the lowest abundance 386 No/L and 134 No/L in the upstream and downstream respectively. There were significant differences in the abundance of these taxa among various seasons ($P < 0.05$) (Table 4). Bacillariophyta has dominated the winter community while the relative abundance ofChlorophyta and Cyanophyta increased. The highest cell abundance of Bacillariophyta was in the winter. Cyanophyta in our study area

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was low to moderate in density and only occasionally dominated the phytoplankton community as reported in the Na Thap river (Lueangthuwapranit *et al.,* 2011). Mixed assemblages had more number of Cyanobacteria were observed occasionally in the upstream. Although Cyanobacteria are known as predominantly freshwater species, our study found that *Microcystis aeruginosa* was present throughout the entire study area. In the summer, Cyanophyta had the maximum abundance, which could be attributed to the increase in water temperature as seen in the Blanes Bay (Hense and Beckmann, 2006).The abundance of phytoplankton differs temporally from wet to dry phase as well as spatially from estuarine to fluvial province (Varona Cordero, *et al.,* 2010). Because of the high abundance of Bacillariophyta, the highest average abundance was detected in winter related to Navicula and Fragilaria. The lowest and the highest cell abundance of Bacillariophyta were reported in summer and winter (359 No/L and 570 No/L, respectively) and their cell abundance showed significant differences at various seasons ($P<0.05$). OfDinophyta, the most and least abundant was in winter (120 No/L) and monsoon (23 No/L) with significant differences among seasons ($P < 0.05$) and the highest abundance was of *Prorocentrum lima*. By contrast, Cyanobacteria had their highest cell abundance in summer (257 No/L) and their lowest was displayed in monsoon (18 No/L), and the highest cell abundance related to Anabaena and Microcystis. There were significant differences in the abundance of these taxa among various seasons ($P < 0.05$). In monsoon and summer, the lowest (49 No/L) and the most cell abundance (156 No/L) of green algae were observed, respectively. And this index exhibited a significant difference between seasons ($P < 0.05$). Euglenoids showed their lowest and the most abundant in winter and summer (210 No/L and 86 No/L, respectively). Charophyta had less abundance than other phytoplankton taxa with a significant difference ($P < 0.05$). Eustigomatophyta had maximum and minimum abundance with 89 No/L and 12 No/L in winter ($P < 0.05$) and monsoon respectively. There were no significant differences in the abundance of Eustigomatophyta during the monsoon. In the end, the highest abundance was observed in the winter, summer, and monsoon seasons that belonged to species Navicula, Anabaena,and Cymbella. The maximum and minimum average abundance of phytoplankton was observed in winter and monsoon respectively. The developments of mixed assemblages of riverine and estuarine species varied seasonally throughout the study period and varied predominantly during the winter followed by summer and monsoon periods when heavy rainfalls regulated the increasing amount of river flow and nutrient runoff from agricultural, aquacultural, and industrial land into the lower estuary. Predominant species in the summer season find more competition for the rich nutrients in the post-monsoon season brought by the floods (Murugan et al., 2019). During the monsoon, because of heavy rain and lower tidal variation, a large influx of fresh water from the rivers carried nutrients, in turn influencing primary productivity in the lower estuary with the original inhabitants replaced by Chlorophytes and Cyanophytes. This compositional structure was corroborating with other estuarine systems of Thailand (Angsupanich and Rakkheaw, 1997). The seasonal rainfall system and tidal cycle patterns lead to the species

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composition in estuaries. In an aquatic ecosystem, growing phytoplankton biomass and increased nourishing state of marine water have a direct association (Bauer et al., 2017).

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Simpson dominance index	3	1.3443	0.4481	0.000822
Shannon-Wiener diversity index	3	3.593	1.197667	0.002661
Margalef Richness index	3	4.313	1.437667	0.009886
Pielou's Evenness Index	3	1.9713	0.6571	0.005976

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	1.907903	3	0.635968	131.4993	**3.81E-07**	4.066181
Within Groups	0.038649	8	0.004831			
Total	1.946552	11				

Table 3: ANOVA: Single Factor for alpha diversity indices

SUMMARY	*Count*	*Sum*	*Average*	*Variance*		
Downstream	3	2552	850.6667	210777.3		
Upstream	3	1821	607	174603		
Winter	2	1623	811.5	5100.5		
Summer	2	2230	1115	71442		
Monsoon	2	520	260	31752		
ANOVA						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	89060.17	1	89060.17	9.260541	**0.033144**	18.51282
Columns	751526.3	2	375763.2	39.07213	**0.024955**	19
Error	19234.33	2	9617.167			
Total	859820.8	5				

Table 4: ANOVA: Two-Factor Without Replication

Conclusion

Chlorophyta, Cyanobacteria, and Euglenophyta were the most common groups in the turbid freshwater habitat (upstream), whereas Bacillariophyta and Dinophyta dominated along the salinity gradient of the clear estuarine environment (downstream). Here, upstream was more productive and diverse than downstream.

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The observed composition of phytoplankton species suggests that important changes have occurred in the phytoplankton assemblages that likely have affected the food web of this estuarine ecosystem. This information is very significant and emphasizes the requirement for continued attempts to implement measures aimed at decreasing nutrient influx into the estuary.

The high phytoplankton abundance was resulting from fluvial influx from surrounding tributaries. The diversity, abundance, and ecology of phytoplankton are governed by the nutrients as well as other water quality factors. As mentioned, increasing the detergents, fertilizers, and nutrients resulting from industrial and agricultural sewages to the water body are important elements for nutrient sources, which regulate the phytoplankton growth and development.

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Article 13

Genotypic Variability in Groundnut (*Arachis Hypogaea* L.) for Tolerance to Foliar Diseases

Meena B,Ushamalini C, Sudhalakshmi C and Praneetha S.

Abstract

Groundnut (*Arachis hypogaea* L.) is an important oilseed crop with high levels of proteins, carbohydrates, vitamins and minerals contained within seeds. Occurrence of groundnut diseases reduces the yield and quality of pods and increases the cost of production.Rust and late leaf spot (LLS) are two major foliar fungal diseases of groundnut and may cause yield loss up to 70%. Use of fungicide is expensive approach and it's not environment-friendly also. Therefore breeding for new cultivars with genetic resistance will be an environmental friendly, sustainable and cost effective approach. Screening and identification of resistant cultivar is one of the primary objectives for groundnut improvement. About forty nine groundnut genotypes were screened for major foliar diseases *viz*., early leaf spot, late leaf spot, rust and *Alternaria* leaf blight under field conditions (hot spot area) using a modified 1- 9 scale. The results of the screening experiment revealed that among the 49 groundnut genotypes screened, seven entries *viz*., ISK-2018-18, ISK-2018-29, IVK 2018-9, IVK 2018-10, IVK 2018-17, IVK 2018-18 and ASK 2018-5 showed resistance to both rust and late leaf spot diseases.

Key words Groundnut, genotypes, foliar diseases, screening

1. Introduction

Groundnut/Peanut is a major oilseed crop widely grown in major tropical and sub-tropical regions of the world. In India, it is primarily an important oilseed crop but growing realization of confectionary quality traits in kernel, it is now being realized as a food and fodder crop. It is valued as a rich source of energy in form of oil (48-50%) and protein (25-28%) in the kernels. It provides 564 kcal of energy from 100 g of kernels (Jambunathan, 1989). Besides this, it contains many health beneficial nutrients such as minerals, vitamins and antioxidants. Groundnut haulms provide nutritious fodder for livestock. It contains protein (8-15%), lipids (13%), minerals (9-17%) and carbohydrate (38-45%) higher than cereal fodder. The major groundnut growing states are Gujarat, Andhra Pradesh, Tamil Nadu, Karnataka, Rajasthan and Maharashtra which contributes about 90 per cent of area and production. Groundnut is cultivated as *kharif* (rainfed or monsoon season) and *rabi-summer* (irrigated) crop and well drained, sandy soils are best suited for production.

The groundnut crop in general experiences several serious biotic and abiotic challenges that limit pod yields. In addition, the yield and quality of groundnut crop will be

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declined due to disease occurrence and increase the cost of production in groundnut growing areas. Among the biotic stresses, foliar fungal diseases *viz*., early leaf spot (ELS) caused by *Cercospora arachidicola* Hori, late leaf spot (LLS) caused by *Phaeoisariopsis personata* (Berk. & Curt.) V.Arx (=*Mycosphaerella berkeleyi* Jenkins) and rust caused by *Puccinia arachidis* Speg. are the most widely distributed and economically important diseases of groundnut and account for more than 50% yield loss (Nataraja *et al.,* 2014). The late leaf spot usually appears at 55 to 60 days after sowing and causes quite 50% loss in pod and haulm yield in groundnut producing areas of Karnataka (Hegde *et al*., 1995; Hegde *et al.,* 2016). The combined infection of rust and leaf spots cause losses to the tune of 90%. Besides reducing pod yield, disease occurrence also affects the fodder quality of haulm. Reduction in pod and haulm yield was reported as 25.3% and 53.0% respectively (Eswara Reddy and Venkateswara Rao, K. 1999). *Alternaria* leaf blight is another fungal disease causing blighting of groundnut leaves. The dry root rot caused by *Macrophomina phaseolina* (Tassi.) Goid, collar rot (*Aspergillus niger*) and stem rot (*Sclerotium rolfsii*) are the main soil-borne diseases in groundnut. However, the incidence orseverity of the disease may vary from season to season. The magnitude of losses due to diseases is high all over the world.

In addition, aflatoxin contamination drastically affects the product quality and greatly reduces value of the crop plant along with grains. Foliar diseases can be controlled by chemical measures but they increase costs of production by 10%, thus beyond the reach of small and marginal farmers and also pollute the environment (Coffelt Ta and Porter, 1986). Considering above facts in mind, development and growing of resistant cultivars is the best viable option to minimize economic losses of farmer and maintains good quality of the product.

Development of disease resistant variety using resistant lines is the main objective for the plant breeders. Also, breeding for disease resistance requires rapid screening techniques which are efficient and low cost also (Foolad *et al*., 2000). Conventional breeding has been the main avenue for providing modern groundnut cultivars to farmers and has been successful in some crops. Hence, the present study was conducted to assess the extent of damage caused by various diseases in peanut genotypes in order to identify the resistant sources for major diseases.

2. Materials and Methods

Field experiments were conducted to evaluate peanut genotypes against major foliar diseases of groundnut *viz*., early leaf spot, late leaf spot, rust and *Alternaria* leaf blight during 2019 and 2020 in *kharif* season at Coconut Research station, Aliyarnagar (identified as hot spot area for foliar diseases of groundnut), Tamil Nadu Agricultural University (Longitude - 77E : latitude - 10N : MSL - 260m). About forty nine peanut genotypes and the susceptible groundnut culivar Co 2 was sown in 30x10 cm spacing in 15 m^2 (5m x 3m) plots. Randomized Block Design was followed for conducting the screening experiments. Three

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replications were maintained for every treatment. Each genotype is flanked by two rows of susceptible check Co 2 on either side so as to create heavy disease pressure in the screening plot. Required intercultural operations were carried out as per the standard package of practices. Early leaf spot, late leaf spot, rust and *Alternaria* leaf blight disease intensity were recorded periodically by adopting modified 1-9 scale as proposedby Subrahmanyam *et al.*,1982 throughout the cropping period (Table 1). The reaction to foliar diseases for each groundnut genotype was assessed as per the following table and the results were furnished in Tables 2, 3 and 4.

Disease scale	Per cent infection (%)	Reaction
1, 2	1-10	Resistant
3, 4	11-20	Moderately resistant
5, 6	21-30	Moderately susceptible
7,8	31-50	Susceptible
9	51-100	Highly susceptible

Table 1. Disease reaction against foliar diseases of groundnut

3. Results and Discussion

Foliar fungal diseases of groundnut cause significant yield loss. Chemical control measures are available but they increase production costs and are beyond the reach of small and marginal farmers as well as they are not eco-friendly approaches. Development of resistant variety is eco-friendly and cost effective approach. Screening and identification of resistant cultivar is one of the primary objectives for groundnut improvement. Germplasm screening is a crucial aspect for identifying resistant lines against plant diseases. The cultures that showed resistant reaction to groundnut diseases will be used as donors in the resistant breeding. However, satisfactory levels of resistance aren't available in cultivable germplasm for these diseases and therefore the present study on identifying elite germplasm lines assumes significance.

The results of the screening experiments conducted during 2019 and 2020 revealed that the groundnut genotypes *viz*., ISK I 2018-18; ISK I 2018-29; IVK I 2018-9; IVK I 2018-10; IVK I 2018-17; IVK I 2018-18; ASK 2018-5 and ASK 2018-9 showed resistant reaction to late leaf spot disease with disease grade of 1 and 2 (Table 2). The genotypes *viz*., IVK I 2018-8; ASK 2018-6 and ASK 2018-7 showed moderately resistant reaction to late leaf spot disease with disease grade of 3 and 4 (Table 2). The susceptible check CO_2 recorded the maximum late leaf spot disease grade of 8 (Table 2). El-Shazly *et al*. (1999) previously reported that germplasm having perfect resistance (without symptom) to wilt disease. Chaudhari *et al*. (2019) documented that combined analysis of variance revealed significant environment (E) and genotype × environment (G×E) interactions for both leaf spot and rust diseases indicating differential response of genotypes in different environments. Nineteen genotypes derived from *A. cardenasii*, five from *A. hypogaea* and

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four from *A. Villosa* showed resistance to leaf spot and rust diseases in various locations (Chaudhari *et al*., 2019).

S.No	Genotypes	Disease severity (1-9 scale)			
		Early leaf spot (ELS)	Late leaf spot (LLS)	Rust	*Alternaria* leaf blight
IVT II (SB)					
1	ISK I 2018-1	1	7(S)	6(MS)	2
2	ISK I 2018-2	1	7(S)	6(MS)	3
3	ISK I 2018-3	1	6(MS)	5(MS)	2
4	ISK I 2018-4	1	6(MS)	4(MR)	1
5	ISK I 2018-5	2	7(S)	5(MS)	1
6	ISK I 2018-6	1	5(MS)	2(R)	1
7	ISK I 2018-7	1	5(MS)	4(MR)	1
8	ISK I 2018-8	1	7(S)	5(MS)	1
9	ISK I 2018-9	1	7(S)	6(MS)	2
10	ISK I 2018-10	1	5(MS)	2(R)	1
11	ISK I 2018-12	1	6(MS)	5(MS)	1
12	ISK I 2018-13	1	7(S)	6(MS)	3
13	ISK I 2018-14	1	7(S)	6(MS)	2
14	ISK I 2018-15	1	7(S)	6(MS)	2
15	ISK I 2018-16	1	7(S)	5(MS)	1
16	ISK I 2018-18	2	2(R)	2(R)	1
17	ISK I 2018-22	2	7(S)	4(MR)	1
18	ISK I 2018-23	1	7(S)	5(MS)	2
19	ISK I 2018-25	2	7(S)	5(MS)	1
20	ISK I 2018-27	2	7(S)	6(MS)	2
21	ISK I 2018-29	1	1(R)	2(R)	1
IVT(VG)					
22	IVK I 2018-1	2	7(S)	5(MS)	2
23	IVK I 2018-2	2	7(S)	3(MR)	1
24	IVK I 2018-3	1	7(S)	4(MR)	1
25	IVK I 2018-4	2	6(MS)	5(MS)	1
26	IVK I 2018-5	1	6(MS)	6(MS)	3
27	IVK I 2018-6	2	6(MS)	5(MS)	1
28	IVK I 2018-7	1	5(MS)	4(MR)	1
29	IVK I 2018-8	2	4(MR)	4(MR)	1
30	IVK I 2018-9	1	1(R)	1(R)	1
31	IVK I 2018-10	1	1(R)	1(R)	1
32	IVK I 2018-11	1	6(MS)	4(MR)	1
33	IVK I 2018-12	1	7(S)	4(MR)	1
34	IVK I 2018-13	1	5(MS)	5(MS)	2
35	IVK I 2018-16	1	5(MS)	6(MS)	2
36	IVK I 2018-17	1	1(R)	1(R)	1
37	IVK I 2018-18	1	1(R)	1(R)	1
38	IVK I 2018-20	1	7(S)	4(MR)	1
39	IVK I 2018-21	1	6(MS)	4(MR)	1
40	IVK I 2018-22	1	6(MS)	3(MR)	1
AVT (SB)					
41	ASK 2018-1	1	7(S)	5(MS)	1
42	ASK 2018-2	1	7(S)	4(MR)	1
43	ASK 2018-3	1	6(MS)	4(MR)	1
44	ASK 2018-4	1	6(MS)	2(R)	1
45	ASK 2018-5	1	1(R)	1(R)	1
46	ASK 2018-6	1	4(MR)	1(R)	1
47	ASK 2018-7	1	4(MR)	2(R)	1
48	ASK 2018-8	1	5(MS)	2(R)	1
49	ASK 2018-9	1	2(R)	4(MR)	1
50	Local Check Co2	2	8(S)	7(S)	3

Table 2. Screening of peanut genotypes for resistance/tolerance to major diseases (Poled mean of 2019 & 2020)

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Identification of disease resistant lines may be a major goal for plant breeders. Screening of groundnut genotypes against rust disease revealed that ISK I 2018-6; ISK I 2018-10; ISK I 2018-18; ISK I 2018-29; IVK I 2018-9; IVK I 2018-10; IVK I 2018-17; IVK I 2018-18; ASK 2018-4; ASK 2018-5; ASK 2018-6; ASK 2018-7 and ASK 2018-8 showed resistant reaction to rust disease with disease grade of 1 and 2 (Table 2). The genotypes *viz*., ISK I 2018-4; ISK I 2018-7; ISK I 2018-22; IVK I 2018-2; IVK I 2018-3; IVK I 2018-7; IVK I 2018-8; IVK I 2018-11; IVK I 2018-12; IVK I 2018-20; IVK I 2018-21; IVK I 2018-22; ASK 2018-2; ASK 2018-3 and ASK 2018-9 showed moderately resistant reaction to rust disease with disease grade of 3 and 4 (Table 2). The susceptible check CO_2 recorded the maximum rust disease grade of 7 (Table 2). Rakholiya and Jadeja, 2010 identified advanced breeding lines for resistance to stem rot and collar rot diseases of groundnut that can be used for inclusion in the further resistance breeding programmes.

Early leaf spot disease grade varied from 1 to 2 among the groundnut genotypes. The disease grade varied from 1 to 3 for *Alternaria* leaf blight disease (Table 2). The susceptible check CO_2 recorded the maximum early leaf spot disease grade of 2 and *Alternaria* leaf blight disease grade of 3 (Table 2). In the present study, much variation was observed for reaction of groundnut genotypes to late leaf spot and rust diseases. Among the groundnut genotypes screened for foliar diseases of groundnut, late leaf spot and rust were found to be predominant diseases in groundnut. Rani *et al*. (2018) identified three groundnut lines (ICGV86699, ICGV91114 and ICGV 89280) for stem rot resistance among the 40 groundnut germplasm screened.

Host plant resistance is one of the effective methods in managing the plant diseases. Identification of resistant sources is a crucial factor in breeding methodology for selecting the resistant donors for incorporation of resistance. In the current research work, four types of disease reaction *viz*., resistant, moderately resistant, moderately susceptible and susceptible reactions were observed among groundnut genotypes (Tables 2, 3 & 4). Screening of groundnut genotypes against foliar diseases of groundnut *viz*., late leaf spot disease revealed that eight genotypes belong to Group I (Resistant reaction with disease grade of 1 and 2); three genotypes belong to Group II (Resistant reaction with disease grade of 3 and 4); seventeen genotypes belong to Group III (Resistant reaction with disease grade of 5 and 6) and twenty two genotypes belong to Group IV (Resistant reaction with disease grade of 7 and 8) (Table 3).

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Groups	Genotype reaction	Disease grade	No. of genotypes	Details of genotypes
Group I	Resistant	1, 2	8	ISK I 2018-18; ISK I 2018-29; IVK I 2018-9; IVK I 2018-10; IVK I 2018-17; IVK I 2018-18; ASK 2018-5; ASK 2018-9
Group II	Moderately resistant	3, 4	3	IVK I 2018-8; ASK 2018-6; ASK 2018-7
Group III	Moderately susceptible	5, 6	17	ISK I 2018-3; ISK I 2018-4; ISK I 2018-6; ISK I 2018-7; ISK I 2018-10; ISK I 2018-12; IVK I 2018-4; IVK I 2018-5; IVK I 2018-6; IVK I 2018-7; IVK I 2018-11; IVK I 2018-13; IVK I 2018-16; IVK I 2018-21; IVK I 2018-22; ASK 2018-3; ASK 2018-4
Group IV	Susceptible	7, 8	22	ISK I 2018-1; ISK I 2018-2; ISK I 2018-5; ISK I 2018-8; ISK I 2018-9; ISK I 2018-13; ISK I 2018-14; ISK I 2018-15; ISK I 2018-16; ISK I 2018-22; ISK I 2018-23; ISK I 2018-25; ISK I 2018-27; IVK I 2018-1; IVK I 2018-2; IVK I 2018-3; IVK I 2018-12; IVK I 2018-20; ASK 2018-1; ASK 2018-2; ASK 2018-5; CO_2
Group V	Highly susceptible	9	--	--

Table 3. Grouping of groundnut genotypes against late leaf spot disease

Similarly, grouping of groundnut genotypes against rust disease revealed that thirteen genotypes belong to Group I (Resistant reaction with disease grade of 1 and 2); fifteen genotypes belong to Group II (Resistant reaction with disease grade of 3 and 4); twenty one genotypes belong to Group III (Resistant reaction with disease grade of 5 and 6) and one genotype belong to Group IV (Resistant reaction with disease grade of 7 and 8) (Table 4). The identified moderately resistant lines may be utilized for breeding programmes to broaden the resistance against major diseases of groundnut. A total of 14 alleles were identified with a mean of 3.5 alleles per locus for polymorphic SSR markers (Bhawar *et al*., 2020).

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Groups	Genotype reaction	Disease grade	No. of genotypes	Details of genotypes
Group I	Resistant	1, 2	13	ISK I 2018-6; ISK I 2018-10; ISK I 2018-18; ISK I 2018-29; IVK I 2018-9; IVK I 2018-10; IVK I 2018-17; IVK I 2018-18; ASK 2018-4; ASK 2018-5; ASK 2018-6; ASK 2018-7; ASK 2018-8
Group II	Moderately resistant	3, 4	15	ISK I 2018-4; ISK I 2018-7; ISK I 2018-22; IVK I 2018-2; IVK I 2018-3; IVK I 2018-7; IVK I 2018-8; IVK I 2018-11; IVK I 2018-12; IVK I 2018-20; IVK I 2018-21; IVK I 2018-22; ASK 2018-2; ASK 2018-3; ASK 2018-9
Group III	Moderately susceptible	5, 6	21	ISK I 2018-1; ISK I 2018-2; ISK I 2018-3; ISK I 2018-5; ISK I 2018-8; ISK I 2018-9; ISK I 2018-12; ISK I 2018-13; ISK I 2018-14; ISK I 2018-15; ISK I 2018-16; ISK I 2018-23; ISK I 2018-25; ISK I 2018-27; IVK I 2018-1; IVK I 2018-4; IVK I 2018-5; IVK I 2018-6; IVK I 2018-13; IVK I 2018-16; ASK 2018-1
Group IV	Susceptible	7, 8	1	CO_2
Group V	Highly susceptible	9	--	--

Table 4. Grouping of groundnut genotypes against rust disease

Though, germplasm screening has been a continuous process against these diseases, integrating the host-plant resistance with other sustainable options under IDM is an ideal strategy over long run.

Conclusion

Foliar fungal diseases *viz*., early leaf spot, late leaf spot, rust and *Alternaira* leaf blight are the major limiting factors for groundnut productivity. Development of disease resistant variety against foliar diseases is cost effective and environment friendly approach also. Resistant variety requires efficient donor for hybridization programme, so genotype screening is a promising approach. Current study identified seven combined late leaf spot and rust resistant genotypes *viz*., ISK-2018-18, ISK-2018-29, IVK 2018-9, IVK 2018-10, IVK 2018-17, IVK 2018-18 and ASK 2018-5 to be used as donors for groundnut hybridization.

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Article 14

Extensive characterization of Java citronella (*Cymbopogon winterianus* Jowitt) for feasible feedstock towards value added production

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Abstract

This study is an endeavor to evaluate the feasibility of citronella biomass to be used for the production of bioenergy and bio-chemicals. The biomass before essential oil extraction (original biomass) and after extraction (spent biomass) were thoroughly characterized in order to compare the efficiency for its use as feedstock for various applications.Besides proximate and ultimate analysis, the physico-chemical analysis of citronella biomass were performed using bomb calorimeter, energy dispersive x-ray spectroscopy, thermo gravimetric analysis, x-ray diffraction, Fourier transform infra-red spectroscopy, scanning electron microscopy, gas chromatography-mass spectroscopy and CHNS. The original biomass was initially hydro-distilled using Clevenger apparatus for essential oil extraction. The citronella oil yield obtained was 2.38 v/w% with high content of commercially important compounds such as citronellal, citronellol and geraniol accounting for 89.14% of the total composition of the oil. Both the biomass, original and spent (after essential oil extraction)were subjected to soxhlet extraction for extractives removal using three solvents viz. hexane, ethanol and water successively, the remaining raffinate biomass was then pretreated using dilute sulphuric acid for production of reducing sugar.Higher amount of extractives was obtained in water indicating more content of polar compounds. Both the biomass has approximately 60% of holocellulose content with higher hemicellulose than cellulose. The supernatant liquid after dilute acid treatment was analyzed for reducing sugar using 3, 5-Dinitrosalicylic acid method and obtained reducing sugar yield of 487.5mg/g of biomass.

Keywords Citronella biomass, Characterization, Crystallinity, Essential oil, Pretreatment, Reducing sugar

1. Introduction

The consumption of energy is increasing in a faster rate due to urbanization and development all over the world leading to depletion of fossil fuels. Lignocellulosic biomass remains the potential alternative energy resource to meet the rising demand. Hence the scope for utilising lignocellulosic biomass to produce energy increases which will help a nation to be self-sufficient in regional and national level. About 220 billion oven-dry ton per year of biomass is available in the world, which is considered the largest source of sustainable energy (Anon, 2004). Additionally aromatic spent biomasses (biomass after essential oil extraction) are the most abundant and underutilized biological resources.These biomasses are non-cattle

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feed and hence discarded as waste orburn haphazardly leading to environmental problems. Indiaalone produces more than 6.0 million tons per annum of aromatic spent biomass (Rout et al., 2013). Lignocellulosic materials can be converted to liquid and gaseous source of energy such as ethanol, butanol etc. and hydrogen respectively.Conversion of lignocellulosic biomass to bioenergy requires pretreatment forhydrolysis of holocellulose to their constituent sugars, fermentation of the pentose and hexose sugars and downstream processing. Out of which pretreatment methods remain the most important steps in order to reduce the recalcitrance of the substrate to achieve high rate of hydrolysis prior to fermentation. Different chemical (acid, alkali, organosolv), physical (ultrasound, milling), physico-chemical (steam explosion, AFEX, liquid hot water) and biological pretreatment (using microorganisms such as fungi and bacteria) methods has been practiced since decade. The efficiency of conversion of biomass to fermentable sugars depends on the choice of biomass and pretreatment technique employed. Besides having high calorific value, ideal biomass for bioenergy production should contain high cellulose and hemicellulose content and comparatively very less amount of lignin, ash and sulphur.

According to APG III system of classification Citronella plant is an aromatic grass, belonging to Plantae kingdom, Clades- Angiosperms, Monocots and Commelinids, Order- Poales, Family- Poaceae, Subfamily- Panicoideae, Tribe- Andropogoneae, Subtribe- Andropogoninae and Genus – *Cymbopogon* Spreng. From the genus *Cymbopogon,* two species are commercially categorized: (1) Ceylon type (*Cymbopogon nardus Rendle)* and (2) Java type (*Cymbopogon winterianus Jowitt)*.The Ceylon type is a perennial, robust, stoloniferous grass of about 1 m height having broad leaves and large compound panicles. The spikelets are awnless, lanceolate and flattened on back. On the other hand Java Citronella is a tufted perennial grass of about 2 m height with stout, erect, terete, leafy node clumps and fibrous roots. Leaf blades are linear, having serrate margins with long acuminate tip. The compound panicle is very large with 20 mm long racemes. The two lower spikelets one sessile and the other pedicelled are homogeneous, male or neuter and the remaining pairs are heterogeneous in both racemes. Ovary is superior, monocarpellary, unilocular and stigma is bifid and leathery (Joy et al. 2001).Java type with about 85% of commercially important compounds like Citronellal, Geraniol and Citronellol is considered more superior to that of Ceylon type with 55-65% of those compounds (Wijesekara, 1973). The Citronella plant grown in North eastern region of India is rich in Citronellal content (Ahmed, 2005). The region experiencing well distributed rainfall all over the year is considered favorable for cultivation of Citronella plant. Sandy loamy soil with high moisture content but without water logging is the appropriate soil condition for the plant growth. The best planting period is rainy season and the most ideal season of harvesting is summer and the early winter. But harvesting during late winter significantly reduces the oil yield (Blank et al. 2007). Citronella oil has a natural insect repellent as well as medicinal properties and is used in fragrance industries such as perfumery, cosmetic, soap, confectionary, flavoring industry etc., and in pharmaceutical

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industry. It has its therapeutic uses as analgesic, anticonvulsant, anxiolytic etc. and is considered a favorable agent towards anti-fungal, anti-bacterial, and anti-parasitic (Almeida et al. 2001, 2003, 2004; Wany et al. 2013).

In order to investigate the composition of citronella biomass for its feasible feedstock for various applications, the physical and chemical characterization of biomass (before and after oil extraction) is carried out using TGA, XRD, FTIR, CHNSO, SEM, EDX, UV-Vis, HPLC and Bomb calorimeter.

2. **Materials and Methods**

2.1. Materials

Citronella plant was obtained from local farms of Karbi Anglong, Assam (India). Whole aerial plants were initially dried in the semi-dark room, chopped into small pieces, and then stored in zipped plastic bags at ambient temperature. After extraction of essential oil, the spent citronella biomass was dried, grinded and sieved to 1mm sizes using 16 BSS mesh screen. Similar procedure was followed for the original biomass for its physical and chemical characterization. Millipore water is obtained using Millipore water synthesis unit bearing Model No: Elix-3, Milli Q; Make: M/S Millipore, USA. 1000 ml capacity 3451-Clevenger apparatus purchased from Borosil was used. All the chemicals used for analysis were obtained from Merck, India. The standard sugars such as glucose, xylose and arabinose were obtained from Himedia and Sigma Aldrich.

1.2.Methods

1.2.1. Proximate analysis of biomass

Moisture contentof the biomass was evaluatedusing oven-dry method. 4 g of biomass sample,placed in crucible was kept in the oven at 103±2 ^{o}C for 24 h. Volatile matter was estimated keeping 1 g of theoven-dried sample in muffle furnace at 925±2^{o}C for 7 min. Ash content was evaluated using 1 g of oven-dried sample in muffle furnace at 600±5 ^{o}C for 12 h. Exact dried weight was measured after the successive cooling and drying process attaining constant weight on keeping the crucible in the desiccator. All the calculations was done on the basis of the ratio between final dried weight and initial weight of the sample taken. Finally the fixed carbon percentage was estimated by means of differences from 100.

1.2.2. Ultimate analysis of biomass

The major organic elements of biomass was estimated in Eurovector EA3000 CHNS-O elemental analyzer. 4 g of biomass sample was used to evaluate the percentage composition of hydrogen, carbon, nitrogen, sulphur and the oxygen was calculated based on the differences from 100.

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1.2.3. Calorific value determination

The calorific value of the biomass was determined using static bomb calorimeter; sealed parr 1108 according to the protocol reportedby Naik et al., 2010.The biomass sample pellets approximately weighing 1 g each was put in contact with the platinum wire attached with cotton thread which was placed in the ignition port. 1 liter of millipore water was added to the bomb then filled up with 20 bar oxygen. The bomb calorimeter was submerged in millipore water and the calorimeter jacket was maintained at 25 ^{o}C by circulating water. In the isothermal jacket, calorimeter was placed with 10 mm air gap between surfaces. The ignition energy when discharged from about 40V platinum wire was evaluated from thevariation in potential throughout a 1256 or 2900 µF capacitor.

1.2.4. EDX analysis

Energy-dispersive X-ray spectroscopic analysis of citronella biomass wasevaluated using Carl Zeiss Sigma VP FE-SEM with Oxford EDS sputtering system electron microscope to determine the percentage weight of the elements present in the biomass. The biomass sample was sprayed over the carbon tape which was glued in the sample stump and gold coating was done to prevent charging. The image of the sample was taken and specific area on the sample was selected to obtain the weight percentage of the elements present in the material. The results obtained were the average of the value of 5 spectrums.

1.2.5. Essential oil extraction procedure

Citronella plant (30 g) and millipore water (500 ml) was charged in the 1000 ml capacity round bottom flask of Clevenger apparatus for hydro-distillation. Heating was provided using a heating mantle (Ikon instruments, Delhi-95). Distillation time was accounted from the moment when the water started boiling and distillation was performed for 3 hrs. The volume of oil was recorded in the Clevenger apparatus at the end of the experimental run. The oil was then collected, stored in an amber vials and kept in the deep freezer at -4 ^{o}C till GC-MS analysis. The oil yield (v/w) was calculated from the ratio of volume of oil collected to the initial mass of the plant material taken.

1.2.6. GC-MS analysis

The qualitative and quantitative analysis of the extracted citronella oil was done using GC-MS on a Perkin Elmer Clarus 680 GC/ 600C MS system. Fused silica HP-5MS column cross-linked with 5% phenyl methyl siloxane having the length of 60m and internal diameter of 0.25 mm was used. The initial oven temp was 50°C for 2 min and then ramp at 5°C/minto 260°C. The hold time was 2 min, and the solvent delay time was 8 min. Helium was used as a carrier gas at 1ml/min. 2 µL volume of oil sample (2% solution of Citronella oil in hexane) was injected. Split ratio 50:1 and scan range of 50 to 600 Da was used. The identification of the constituents was done on the basis of mass spectra library search (NIST). The relative amount of individual components was evaluated according to the GC peak area.

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1.2.7. Extractives determination

The amount of extractives in citronella biomass was estimated using NREL protocol using hexane, ethanol and water sequentially. The biomass was extracted using soxhlet apparatus for 12 h and the solvent after subsequent extraction was separated using rotary evaporator. The weight of the extractives was measured and percentage extraction was calculated from the ratio of final weight obtained to the initial weight.In order to extract the non-polar compounds such as lipids, hydrocarbons, terpeniods etc. the biomass was first extracted using hexane. The raffinate biomass after extraction with hexane was further extracted using ethanol to remove the compounds such as waxes, chlorophyll, sterol etc. from the biomass. The biomass was later extracted using Millipore water to removenon-structural carbohydrates and inorganic materials. The final biomass after extraction with soxhlet was hydrolyzed with dilute acid to determine the quantity offermentable sugars in the biomass. Thermo gravimetric analysis (TGA) of the final raffinate was performed to estimate the amount of hemicellulose, cellulose and lignin.

1.2.8. TGA analysis for Hemicellulose, Cellulose and Lignin content

Thermo gravimetric analysis helps to study the degradation profile of the material and its kinetics with respect to temperature. Researchers have reported that degradation of biomass samples follows 1st order reaction kinetics and hence the concentration of the individual constituents in a biomass is directly proportional to the degradation rate (Ledakowicz and Stolerak, 2002, Saddawi et al., 2009).To determine the major chemical composition of lignocellulosic biomass viz. hemicellulose, cellulose and lignin, TG analysis was performed using NETZSCH TG 209 F1 Libra® at the heating rate of 10 ^{o}C/minfrom 30 ^{o}C to 700 ^{o}C under nitrogen atmosphere.

1.2.9. Pretreatment of biomass

Pretreatment of a lignocellulosic biomass is necessary before enzymatic hydrolysis to provide easy access for enzymes to convert cellulose to chains of glucose and its isomers.In this study, 0.2g biomass sample on dry basis was treated using 4ml of 0.1M sulfuric acid in a test tube at 120^{o}C and 120min. Care was taken to ensure that the biomass was completely wetted by mixing for 5-10 min before the reaction. After reaction, the sample is allowed to cool and pipetted out specific volume (0.1 µl) of the liquid fractions to determine the concentration of reducing sugars using DNS method. Triplicate experiments were performed and the average value is reported.

1.2.9.1. DNS analysis

3, 5-Dinitrosalicylic acid method was used to determine the amount of reducing sugar as reported by Miller, 1959. In order to prepare 100 ml of DNS reagent, 1.6 g of NaOH was dissolved in 80 ml of distilled water, then 1g of DNS was dissolved to the above solution. 30 g of sodium potassium tartrate was added and the final volume was made up to 100 ml. To estimate the amount of reducing sugar present in the sample,0.1ml of sample was taken in a

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test tube and distilled water was added to make up the volume of 1ml, later 3 ml of DNS reagent was added and placed in boiling water for 5min. After cooling, 6 ml of distilled water was added to the test tubes and mixed well. Then the samples were analyzed inUV-Vis (Model: lambda 45, Perkin Elmer) at 540nm. The concentration of reducing sugar were determined from the calibration plot prepared for standard glucose sugar.

1.2.10. FTIR analysis

Potassium bromide (KBr) and powdered biomass sample were ground well for proper mixing and directly taken for the Fourier Transform Infrared Red (FTIR) analysis. The functional groups present in citronellabiomass were determined by comparing the vibrational frequencies with respect to % transmittance and wave number obtained from FTIR spectrometer "IR Affinity1" (Shimadzu Corporation, Japan), measured at the range between 4000-400 per cmwith resolution of 2 percmand 30 scans per sample.

1.2.11. XRD analysis

The crystallinity of the biomass was determined using wide angle X-ray diffractiometer (Bruker D8 Advanced X-ray diffraction measurement systems) at the radiation generated with 40kv and 40 mA. The diffraction angle (2θ) scan range was 10-28^{o}at a scan speed of 1˚/minand step size 0.05˚.Crystallinity Index was calculated based on the intensity of amorphous region at ~18.5^{o}of 2θ (I_{amp}) and the maximum intensity obtained from crystalline fraction at ~ 22.1^{o}of 2θ (I_{002}) by using the following formula.

$$CrI\ (\%) = \frac{I_{002} - I_{amp}}{I_{002}} \times 100 \qquad (1)$$

1.2.12. SEM analysis

The samples were attached to glued conductive carbon tape on aluminum sample holder. As the sample was non-conducting, so sputter gold coating was performed to prevent charging. All the specimens were examined using a LEO 1430VP scanning electron microscope, under vacuum condition at accelerating voltage of 8kV and 10kV, and the working distance of 15 mm. The image was taken at the magnification of 1.78 KX.

3. Results and Discussions

3.1. Proximate and Ultimate analysis

Proximate and ultimate analysis of a biomass is an efficient conventional way to evaluate the quality of the raw material for biofuel and biochemical production.Higher moisture content leads to decomposing during storage which will result to loss of energy and accordingly reduces the conversion efficiency of a biomass. Volatile matter are mostly in the form of gas and hydrocarbons which are more readily devolatilizing than solid fuel. The

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higher volatile mater and relatively less fixed carbon contentof a biomass is effective for utilizing into gasification and pyrolysis. The ash content in a biomass is an inherentconstituent of plant material comprising mineral matters such as salt of silica, potassium, zinc, calcium, copper, magnesium and manganese,havingenzyme inhibition effect which reduces the hydrolysis of cellulose into fermentable sugars. The ultimate and proximate investigation of the biomass are given in Table 1.CHNS-O analysis of the biomass shows higher content of carbon, hydrogen and oxygen with low nitrogen content and almost negligible sulphur content. Low sulfur content signifies less SO_x emission during gasification unveiling a potential feedstock for gasification. Original biomass has a higher carbon, hydrogen and sulphur but lesser nitrogen and oxygen content than spent biomass. Calorific value is inversely proportional to moisture content of biomass (Ezeike, 1984). The calorific value of around 17 MJ/Kg for both the original and spent citronella biomass is comparable with other biomasses used for energy production studies such as barley straw (15.7 MJ/Kg), flax straw (17.0 MJ/Kg), timothy straw (16.7 MJ/Kg) reported by Naik et al., 2010. Abdullah et al., 2010 accounted for rice husk as 14.79 MJ/Kg and paddy straw 13.74 MJ/Kg whereas Sasmal et al., 2012 reported 17.83MJ/Kg for areca nut husk.The low ash content and sulphur content with good calorific value suggests that citronella biomass can be used for energy generation.

Biomass Name	**Proximate Analysis (%)**				**Ultimate analysis (%)**					**Calorific value (MJ/Kg)**
	Moisture (%)	**Volatile matter (%)**	**Ash (%)**	**Fixed Carbon (%)**	**Carbon (%)**	**Hydrogen (%)**	**Nitrogen (%)**	**Sulphur (%)**	**Oxygen (%)**	
Original Citronella	9.47 ± 0.14	85.30 ± 0.21	0.39 ± 0.02	4.84 ± 0.11	43.74 ± 0.04	5.94 ± 0.07	2.20 ± 0.11	0.3 ± 0.02	47.82 ± 0.14	17.01± 0.26
Spent Citronella	10.06 ± 0.08	82.77 ± 0.33	0.57 ± 0.11	6.20 ± 0.17	42.55 ± 0.10	5.83 ± 0.03	3.44 ± 0.13	0.1 ± 0.01	48.08 ± 0.12	16.98± 0.18

Table 1.

Proximate and Ultimate analysis of Citronella biomass.

3.2. Elemental analysis using EDX

Besides CHNS-O, the other elementspresent in the biomass were determined using EDX as represented in Table 2. The original biomass contains higher amount of K, Mg, Ca, Cu, Si, Cl, P, F and Fe but lesser amount of Al and tungsten. Those elements are mostly the uptake of plants from soil and atmosphere. The presence of those elements in a biomass results to inhibition effect during enzymatic hydrolysis. The lesser content of those elements in the spent biomass than original biomasssuggests that spent biomass would be easier for enzyme to hydrolyze compared to original biomass.

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Citronella Biomass Type	Elements (Weight %)											
	K	Mg	Ca	Cu	Si	Cl	P	F	Fe	Co	Al	W
Original Biomass	1.15 ± 0.05	0.43 ± 0.27	0.63 ± 0.28	0.55 ± 0.05	3.86 ± 0.16	1.0 ± 0.01	0.56 ± 0.37	0.65 ± 0.05	0.3 ± 0.1	0.1	0.5 ± 0.1	0.6 ± 0.26
Spent Biomass	0.15 ± 0.05	0.26 ± 0.14	0.33 ± 0.17	0.23 ± 0.11	1.05 ± 0.15	0.2 ± 0.1	0.1	0.1	0.2	0.1	0.9 ± 0.8	1.05 ± 0.65

Table 2. EDX analysis for some common elements

3.3.GC-MS analysis

The composition of hydro-distilled oil was determined on the basis of peaks obtained in the GC-MS (Fig. 1)as given in Table 3. The first major peak obtained was Citronellal at 26.94 min revealing citronellal as the most volatile component in citronella oil. Silva et al., 2011, and Manaf et al., 2013, in their study also reported Citronellal as the first major peak. The presence of two peaks for Geraniol at 29.24 min and 29.27 min may be due to the presence of its isomer nerol as discussed by Madivoli et al., 2012.

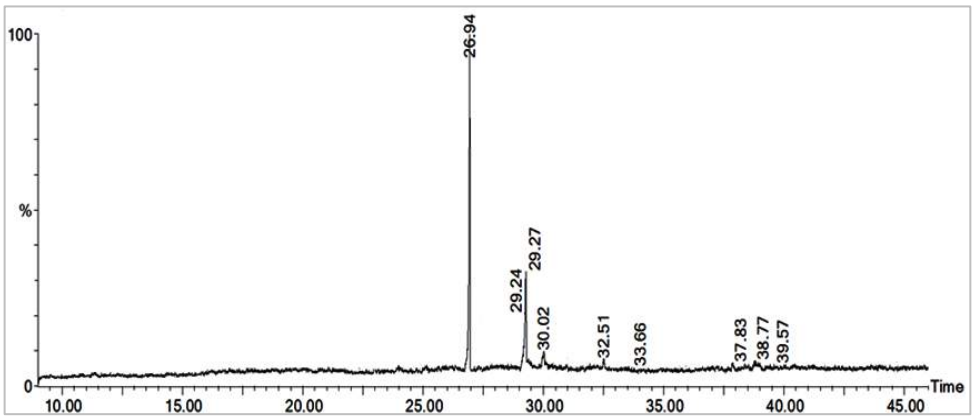

Fig. 1. GC-MS chromatogram of citronella oil extracted by hydro-distillation

The chromatogram plot obtained revealed a high concentration of commercially important major three compounds viz. Citronellal (48.39%), Geraniol (24.86%) and Citronellol (15.89%) accounting for 89.14% of the total constituents. Li et al., 2013 accounted Citronellal- 37.51%, Citronellol- 15.12% and Geraniol- 22.57% totaling to 75.20% of the Java Citronella oil. Beneti et al., 2011 obtained sum total of 71.32% constituting Citronellal (40.23%), Citronellol (13.39%) and Geraniol (17.70%). Pinheiro et al., 2013, also reported composition as Citronellal-23.62%, Citronellol-17.10% and Geraniol-28.62% amounting 69.34% of the total composition. About 68% of the total composition of the Citronella oil comprising Citronellal(30.59%), Citronellol (19.30%) and Geraniol (18.17%) was reported by Songkro et al., 2012 whereas Cassel and Vargas in 2006 reported Citronellal (35.90%),

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Citronellol (5.2%) and Geraniol (20.90%) quantifying 62% of the total composition of the oil. From this analysis it is clear that the hydro-distilled citronella oil is of highly superior quality. Differences in the composition of the oil may be driven by various factors such as variation in genetical makeup, geographical locations and environmental conditions (Sarma et al., 2001; Sarma, 2002).

Retention time (min)	Compound name	Abundance %
26.94	Citronellal	48.39
29.27	Citronellol	24.86
30.02	Geraniol	15.89
31.69	3-Hydroxy-5-isopropyl-2-methylbenzo-1,4-quinone	0.11
32.51	Citronellylpropanoate	3.81
33.66	Limonene	0.10
34.14	β-Elemene	0.07
35.35	Patchoulane	0.08
35.59	Linalool	0.07
36.92	Germacrene D	0.09
37.83	δ-Cadinene	0.11
38.77	Germacrene A	0.15
39.57	Germacrene D-4-ol	0.13

Table 3.Chemical composition of Citronella oil identified using GC-MS

3.4. Extractives estimation

The determination of composition of structural carbohydrates and lignins in a biomass is highly affected by the extractives content. Extractives in biomass consists of non-structural aromatic compounds which includes volatile oils, chlorophyll, fatty acids and their esters, waxes, resins, tannins, terpenes etc. and can be used for production of green chemicals (Sasmal et al., 2012). These materials are either polar or non-polar which can be extracted by using different solvents based on their polarity. So to extract those constituents from citronella biomass, hexane was used to remove non-polar compounds such as lipids, hydrocarbons and terpenes, followed by ethanol to extract polar compounds such as chlorophyll, waxes and sterols and finally by water to remove the non-structural sugars and other inorganic compounds. Hexane for both the biomass (3.62 and 3.06) has higher extract yield than ethanol (2.95 and 1.79) but lesser yield than water (7.46 and 6.68) unveiling the presence of more polar compounds in citronella biomass.

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Citronella Biomass Type	Hexane extract	Ethanol extract	Water extract	Hemicellulose	Cellulose	Lignin
Original Biomass	3.62 ± 0.13	2.95 ± 0.21	7.46 ± 0.17	32.66 ± 0.23	31.70 ± 0.11	18.97 ± 0.14
Spent Biomass	3.06 ± 0.11	1.79 ± 0.15	6.68 ± 0.26	32.22 ± 0.28	28.15 ± 0.12	18.78 ± 0.19

Table 4. Extractives (g) from 100 g biomass sample.

Estimation of hemicellulose, cellulose and lignin, which are the major chemical components of lignocellulosic biomass are important in order to determine the conversion efficiency of biomass into bioenergy and valuable chemicals.It is observed that citronella biomass has lesser cellulose content (31.70 % in original biomass and 28.15 % in spent biomass) than hemicellulose (32.66 % in original biomass and 32.22 % in spent biomass).The lignin content is approximately same (~19%) in both case ofthe biomass (Table 4). Rolz et al., 1986 reported 30 % hemicellulose, 28.5 % cellulose and 11.1 % lignin in java citronella bagasse.

3.5. TGA analysis

The degradation nature of the lignocellulosic biomass can be characterized by the intensity of degradation. Prominent slope of TG curve can be observed at 200-500^{o}C, which represents the reduction of weight due to degradation of hemicellulose, cellulose and some lignin. The efficiency of biomass chemical conversion depends on the total amount of each of these components. The weight loss profile can be described as the loss of easy volatile upto 100^{o}C, loss of water from 100-130^{o}C, 130-200^{o}C shows loss for volatile compounds, 200-350^{o}C for hemicellulose, 300-375^{o}C for cellulose and steady decomposition of lignin from 200-500^{o}Cdue to its more thermally stable nature in contrast to hemicellulose and cellulose (Naik et al., 2010, Carrier et al., 2011).

To get a better picture about the degradation of biomass, TG analysis of standard xylan and cellulose was performed. The onset temperature for xylan and cellulose were observed at 200 ^{o}C and 250 ^{o}C respectively. Degradation range of xylan is 200-320 ^{o}C, on the other hand cellulose degrades from 250-375^{o}C.Xylan has two discrete peaks with its peak temperature at 242 ^{o}C and 284 ^{o}C whereas peak temperature of cellulose was observed at 340^{o}C. The third peak of xylan and second peak of cellulose might be due to impurities or presence of minuscule lignin. Both the biomass followed the same trend with three distinct

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peaks showing the degradation of hemicellulose, cellulose and lignin (Fig. 2). Original biomass has peak temperature of 297 ^{o}C, 330 ^{o}C and 428 ^{o}C whereas the spent biomass has peak temperature at 309 ^{o}C, 333 ^{o}C and 433 ^{o}C, corresponding to the temperature of maximum degradation for hemicellulose, cellulose and lignin respectively. With respect to the degradation range obtained for the standard xylan and cellulose, the relative percentage for cellulose hemicellulose and lignin content of the biomass was estimated. Both biomass has approximately same content of those composition with slightly lesser cellulose content in spent biomass, which may be due to the dissolution of easy soluble holocellulose content during hydro-distillation at boiling temperature of water.

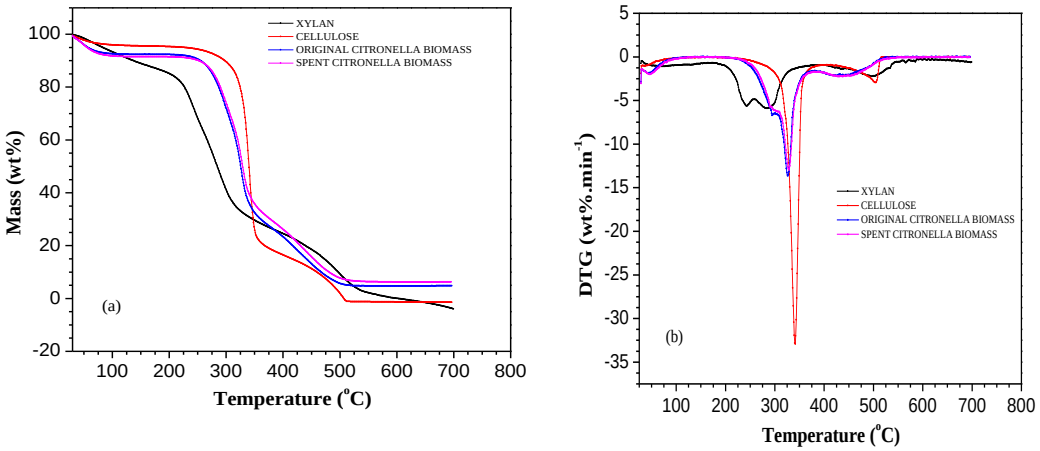

Fig. 2: Thermal analysis of biomass and standard samples; (a) TGA and (b) DTG.

To understand more inside of the thermal behavior of biomass researchers emphasize on the temperature of 50%weight loss; T_{50} (^{o}C)and reactivity of biomass or rate of weight loss at T_{50};R_{50} (%/min). The higher value of R_{50} will signify how fast the decomposition of the biomass takes place or how reactive the biomass is at T_{50} (Abdullah et al., 2010). The data at 50% weight loss for citronella biomassis depicted in Fig. 3. From the comparative analysis of T_{50} and corresponding R_{50}, It was observed that Xylan has least T_{50} (283.17 ^{o}C) and highest R_{50}value (13.89 wt%/min). On the other hand standard microcrystalline cellulose has 342.86 ^{o}C and 8.56 wt%/min T_{50} and R_{50} value respectively. The higher R_{50} value of spent citronella biomass (8.27 wt%/min at T_{50}:333.04 ^{o}C) than original biomass (6.86 wt%/min at T_{50}:328.57 ^{o}C) signifies that the spent biomass is more reactive at T_{50}, which implies that the spent biomass is more feasible to be used for pyrolysis and gasification process. Sasmal et al., 2012 has reported T_{50} and R_{50} value in the range of 320-356 ^{o}C and 7.2-7.4 wt%/min respectively for areca nut husk, bonbogori and moz biomass. Abdullah et al., 2010, found T_{50} and R_{50} value in the range of 363-410 ^{o}C and 2.9-3.19 wt%/min for rice husk, paddy straw, oil palm shell and oil palm frond on their studies for potential biomass for hydrogen production.

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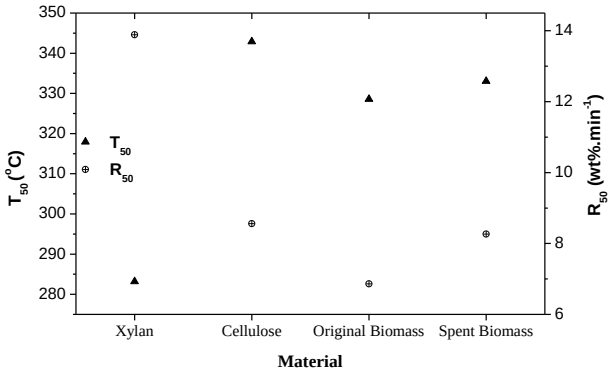

Fig. 3. T_{50} and R_{50} profile of biomass and standard samples

3.6. FTIR analysis

FTIR of original and spent citronella biomass was performed to compare the distribution of functional groups present(Fig. 4).An intense strong and sharp peak in the range of 3600-3200 per cmwas observedin both samples which corresponds to polymeric free hydroxyl (O-H) stretch.Another intense and bifurcated peak in the range of 2935-2915 per cmcorresponding to the C-H Methyl and Methylene asymmetric stretch which are mostly aliphatic alkyl group was observed. A characteristic medium peak is observed at 2353.16 per cmin pre hydro-distilled biomass which may corresponds to certain cyanocarbons. Another distinct and sharp peak observed in the range of 1750-1705 per cmin both the samples signifies un-conjugated xylan as well asaldo, keto, estero and or acido (C=O) stretch. Only the dried original biomass depicted a medium peak at 1504.48 per cm corresponding to aromatic C=C ring and lignin ring stretch. Methylene C-H (1485-1445 per cm), Methyl C-H symmetric bend (1380-1371 per cm) in cellulose and hemicellulose and aryl-O-H stretch (1270-1230 per cm) are present in both the samples. Moreover C-O bend (1140-1050 per cm), Simple –OH stretch (1200-1100 per cm) and CH=CH trans-unsaturated (910-860 per cm) functional groups with medium peaks in both samples are observed. Minor vibrations in the range of 750-660 per cmattributing aromatic, Vinyl C-H group are also seen in both the samples. The wavenumber in the range of 3500-2500 per cmand 1700-550 per cmare generally due to the glycosidic bond of cellulose or the syringyl, hydroxyl phenyl and guaiacyl groups of lignin whereas the band in the range of 1700-1500 per cmcorresponds to aromatic ring stretch of lignin on the other hand the peak at ~1450 and ~894 per cmrepresents the crystalline and amorphous fractions of cellulose respectively (Sasmal et al., 2012).

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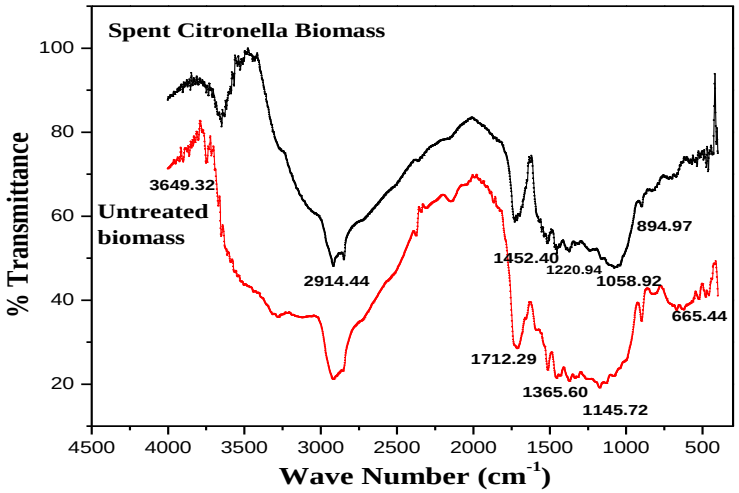

Fig. 4. FTIR analysis of Citronella plant

3.7. Acid pretreatment of spent citronella biomass

Extraction of fermentable sugars from original biomass is not viable since it contains the valuable essential oil so only the spent biomass was used for sugar production. To estimate the amount of sugar extractable in the dilute acid pretreatment process, citronella biomass sample was treated using dilute sulfuric acid and the supernatant liquid was analyzed using DNS method. Sodium potassium tartrate (Rochelle salt) increases the ion concentration in the solution and reduces the tendency to dissolve oxygen. Sodium bisulphate is used to stabilize the color and to react with any oxygen present. NaOH solution is used for the redox reaction between reducing sugars and DNS (Miller, 1959).487 mg of reducing sugar was obtained when 1 g of spent biomass was pretreated using 0.1M sulfuric acid at 120ºC and 120 min reaction time. These good amount of reducing sugar obtained from the biomass projected the viable utilization of spent citronella biomass for the production of biofuel such as bioethanol or biobutanol.

3.8. XRD analysis

The strong hydrogen bonding between the cellulose chains and van der waals forces between the glucose molecule in cellulose and hemicellulose makes the lignocellulosic biomass crystalline reducing the enzymatic hydrolysis of complex polymeric molecule resulting to low fermentable sugar yield. To understand the morphological change with respect to the untreated, hydro-distilled and acid treated biomass, X-ray diffraction analysis was performed. Changes in crystallinity of biomass exhibits due to the cellulose, wax and complex bonding nature of holocellulose and lignin (Naik et al., 2010). The higher peak intensity signifies the higher crystallinity of the sample. The biomass crystallinity increases

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with subsequent pretreatment revealing the disruption of lignin structure and exposure of cellulose on the surface of the biomass successively. The original biomass before undergoingany pretreatment process has the crystallinity index value of 32.97%. When the biomass was hydro-distilled the crystallinity value increases to 34.91% implying the removal of water extractives in the hydro-distillation process.The maximum crystallinity of 55.46% was observed at the acid pretreated sample which shows that the pentose sugars of hemicellulose and minuscule lignin was removed in the acid treatment process increasing the crystallinity of cellulose content (Fig. 5).

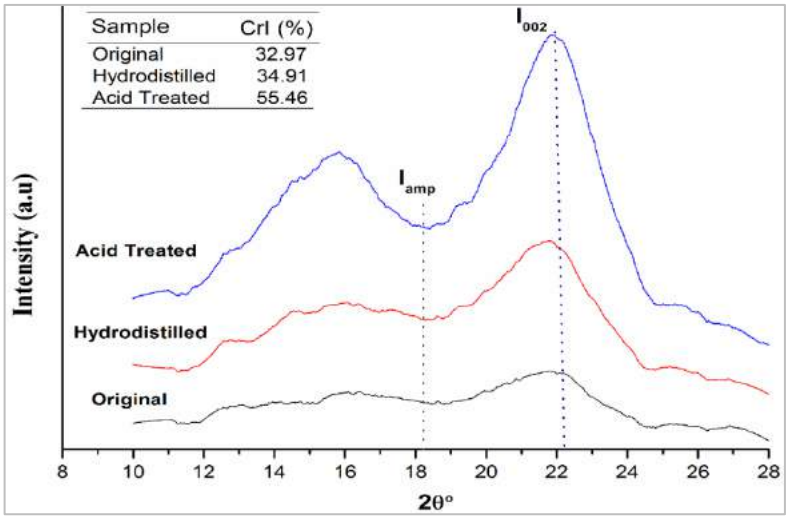

Fig. 5. XRD analysis of Citronella plant

3.9. SEM analysis

SEM analysis of citronella plant sample shows a significant change in the structure after hydro-distillation and acid pretreatment. The cells of the untreated plant material are intact and a smooth surface can be observed from Fig. 6(a). When the plant material is hydro-distilled the cellular content of the plant tissues gets distorted due to extensive thermal stress on the oil glands causing the rapid expansion of oil glands causing to disruption andreleasing the oil content as represented in Fig. 6(b).The morphological changes in the acid treated sample as depicted in Fig. 6(c) reveals the removal of significant amount of reducing sugars and lignin(Giese et al., 2013)

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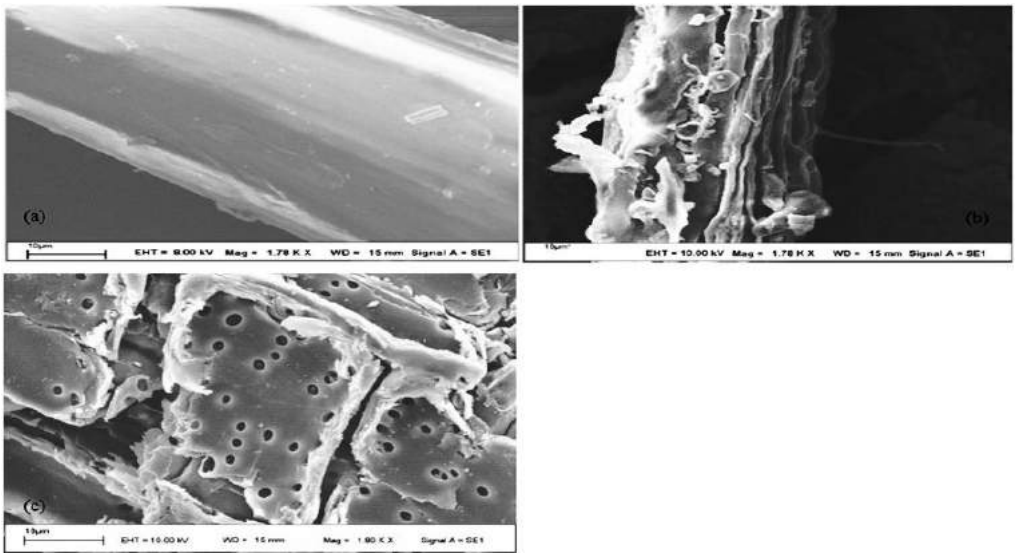

Fig. 6. Electron micrograph of Citronella plant; (a) Original biomass (before hydro-distillation) (b) Spent biomass (after hydro-distillation) and (c) after acid pretreatment.

Conclusion

This work has evaluated and projected the commercial significance of citronella biomass. Besides the important essential oil content, citronella biomass remains a potential feedstock for the production of bioenergy and value added products due to its high holocellulose content. Likewise the high calorific value, high volatile matter with high content of carbon, hydrogen and oxygen and low ash and fixed carbon with negligible suphur content in the biomass obtained in the proximate and ultimate analysis reveals an ideal biomass for energy production.Apart from essential oil content, the original citronella biomass also contains more elements compared to spent biomass suggesting more feasibility of the spent biomass to be used for hydrolysis to extract sugar contents. The application of citronella biomass in the production of different products is to be assessedin further studies in order to give a clear picture as possible feedstock.

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Article 15

Diversity of Some Ethnomedicinal Plants in Kunkuri of Jashpur District

Sangeeta Yadav and Lata Sharma

Abstract

Diversity and ancient health care system are inseparable for each individual in developing countries. 40 % of the drugs sold worldwide contain bioactive compounds derived from plant material. Plants are used since time immemorial to heal and cure various types of diseases; to enhance health care system and for the wellbeing of human kind. The importance of traditional knowledge, potentiality of medicinal plant and future challenges of the ethno medicinal plant bioprospecting have been mentioned in this paper. The local inhabitance of the forest has the knowledge of the medicinal plant and they treat various ailments. But on the other hand the agricultural growth and disinterest of the young people to know about the ethno medicinal plants and traditional knowledge have become a treat. So it is urgency to document and to preserve such plants. Further it requires proper identification of plant species with its herbal values and preparation. To save beneficial plants from additional loss, involving native communities in the cultivation of the foremost utilized medicinal plants is usually recommended. This paper reports an associated study that centered on the standard medicinal plants employed by the local communities to treat human diseases. Study of beneficial medicinal plants were documented from 2018-2020, in the Jashpur district. Ethno medicinal information was collected by guided field works and observations. A complete of twenty medicinal plants, distributed in families, were collected and known. Plant family with the highest medicinal values within the study area, used for diverse disease treatment was family Leguminosae (15.8%).

Keywords Diversity, Ethno-medicinal plants, Ancient medication, Jashpur, Content, Documentation.

1. Introduction

Diversity makes a vital role in creating goodness of human beings and maintains all things that are essential for the balancing of the eco- system. The whole geographical area among the districts of Jashpur is 6457.410 Square Kilometres. Of that total forest area is 2752.285 sq. km. that is forty two.622%of the full geographical area. The soil in higher ghat is lateritic, whereas in Niche ghat is Sandy & Sandy dirt. The Jashpur district covers the major portion of Sal forests. multiethnic teams inhabit in this district. The area is several divides into district topographic structure i.e. upland name higher stairway and Lowland referred to as lower ghat. The standard communities living within the region embrace Oraon, Kanwar, Korwa, Rawotia, Lohar, Ghasi, Painkra, Khariwar, Gasiya, Domra, Tori, Nagesiya,

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Mahakul, and Barga Oraon. There are several tribal groups residing among sensible forest and distinctive topography. Plants are a part of the human culture since pre-historical times. Moreover, ancient medicinal plant information is priceless for contemporary discovery, and lots of trendy medicines were developed from such traditional knowledge. Consequently, an awfully vital a part of the documented ethnomedicinal data relies on studies of the Jashpur district. Ethno-botany deals with the foremost standard commonly are the study and utilization of ethnomedicine. Ethnomedicine involves the study of indigenous beliefs, concepts, knowledge, and practices among the ethnic teams of tribal and rural individuals for prevention, and treatment. Traditional medicines play an economical role in the preparation of herbal medicine for the betterment of individuals. This technique of medicines is employed for hardening diseases through the forces of nature. Tribal individuals have their own system of medicines, that are old, and a few of them aren't documented within the literature. Various components of medicinal plants are considered the natural gift for humans. To cure jaundice and diseases, herbs, shrubs, and trees are used for its. Like an inflammatory, diaphoretic, drug, etc. attributable to the geographical position and difficulty suggests that of communication, people of some major tribes of Jashpur district largely live in villages and rural areas and belongs to various cultures. Ethnomedicines have created sensible contributions in the health care system in traditional medicines for the treatment of several diseases since the past. Ethnomedicinal plants are typically used for curing varied ailments like diabetes, dysentery, typhoid, and fever. Different components of the plant, as well as roots, leaves, fruits and flowers square measure used for the treatment of jaundice. Ethnomedicinal plants like Tulsi, Neem, Turmeric, Giloy, Papaya cure many common ailments. These are thought-about as home remedies in several components of the country.I is understood proven fact that numerous customers are exploitation Basil (Tulsi) for creating medicines, black tea, in pooja and different activities in their day to day life. In many components of the plant, several herbs are accustomed honor their kings showing it as an image of luck. Now, when finding the role of herbs in medication, numerous customers started the plantation of Tulsi and different ethnomedicinal plants in their home gardens. except for the healthful uses, herbs are employed in natural dye, pest management, food, perfume , tea so on. In several countries, totally different styles of medicative plants/ herbs are accustomed to keep ants, flies, mice, and feet far away from homes and offices. currently a day's ethnomedicinal herbs square measure necessary sources for pharmaceutical manufacturing. Over the past twenty years, there has been an incredible increase in the use of flavoring medicine; but, there's still a significant lack of analysis information during this field. Vegetation surveys determine the plant species in every website, what percentage of species are a gift, and also the fraction of the full website space every species coated. The latter 2 are spoken as species diversity and % cover severally. Diversity is an association of all living components of the worldwide diversity. Finally, it's over that the variety of the plants are a serious element of nature not solely necessary for his or her worth as a supply of energy. A plant provides a higher setting and platform to all or any the living beings in the healthy development of the character.

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Diversity is an association of all living components of the character from totally different possible natural habitats.

2. Material and Methods

3.1. Study area

The study was beneath taken by completing ethno-botanical survey with the individuals living within the space under study. The current study was conducted throughout season of 2018-2021 within the Jashpur district of Chhattisgarh. The climate of the realm is tropical with temperature is starting from 35°c to 12.4°c and annual precipitation is three hundred and sixty five days millimeters. The species were ascertained and known with the assistance of native of villages within the forest area and forest guards. Finally, plants were documented of following their biological science name, family, habits.

3.2. Ethnomedicinal documentation

The ethno-medicinal data was documented and field visits were additionally conducted for the gathering of the specimen. A questionnaire based mostly survey was conducted to document ethno-medicinal data of the local and tribal individuals of the area.

3.3. Data analysis

All the species were listed in alphabetical order by their scientific name, family, local name, general name, plant components used, habit.

3.4. Identification of plants

The location and type (shape) of the plant can also be accustomed provide characteristic clues. Plant identification uses anatomical and morphological clues to check best-known plants with unknown plants. Name, components used, and uses of the individual plants.

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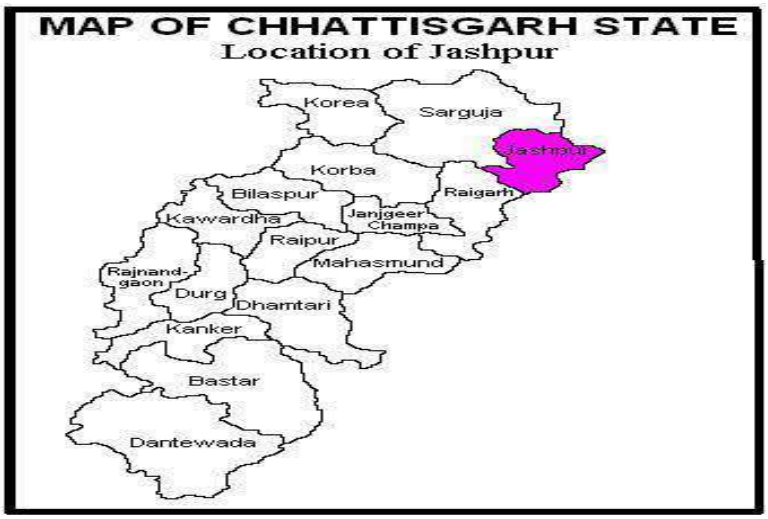

Figure-1: Map of Jashpur District

4. Results And Discussion

A total variety of twenty plant species happiness to varied families (16) with totally different habits were recorded (Table-1). Out of those plants species, shrubs (4), herb (4) and Tree (12) were noticed. Most plant species were recorded for family Leguminosae and Rutaceae family sixteen family. Ascertained plant species with their uses are listed in Table-1., Table-2 shows the family wise distribution of plants and Table-3, the quantity of various habits is given. Vegetation of a as system ends up in and regulates the method of property of the system. Diversity of plants includes their presence and habit in specific ecological space. Tropical moist space includes wealthy species diversity. Further, data of plant diversity is required for the study of dynamic nature of vegetation beneath specific eco-environment state of affairs. The analysis of healthful plants has had a protracted history, and particularly with relation to assessing a plant's quality. Result additionally showed outstanding use of freshly harvested plant components for ancient remedy preparation used against numerous ailments. People of the study area harvest different plant parts for the preparation of traditional remedies (e.g., seeds, barks, leaves and fruit). This is because it is believed that roots contain more concentration of active ingredients.This observation is a good indication of the fact that the local people have not yet started cultivating most of the plant species they are using as remedies. However, other findings indicated that herbs were the most frequently used plant categories. The highest number of remedies is documented for diabetes problem (20%) which are followed by skin health and digestion (18%)

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S.No.	Local Name	Botanical Name (Plant species)	Family	Habit	Parts	Uses
1	Bael	*Aegle mormelos(L.)*	Rutaceae	Tree	Fruit, leaf	Scurvy, digestion
2	Neem	*Azadirachta indica A.juss*	Maliaceae	Tree	Leaf, flower, stem	Fever, diabetes
3	Kajoo	*Anacardium occidentale L.*	Anacardiaceae	Tree	Fruit	Blood pressure, good for heart
4	Bhuineem	*Andrographis paniculata*	Acanthaceae	Herb	Leaf, root	Cold, fever
5	Kachnar	*Bauhinia variegate*	Fabaceae	Tree	Leaf, stem	Control blood sugar purification
6	Mirch	*Capsicum annum*	Solanaceae	Shrub	Fruit, leaf	Digestion, helps in weight loss
7	Papita	*Carica papaya*	Caricaceae	Tree	Fruit	Nerve pains, elephantoid
8	Sadabahar	*Catharanthus roseus*	Apocynaceae	Herb	Flower, leaf	Diabetes, skin health
9	Haldi	*Curcuma longa*	Zingiberaceae	Herb	Tuber	Digestion, ulcers
10	Nimbu	*Citrus limonum*	Rutaceae	Tree	Fruit, bark	Lose weight, digestion
11	Shisham	*Dalbergia sissoo*	Fabaceae	Tree	Leave, bark	Fever, skin diseases
12	Peepal	*Ficus religiosa*	Mulberry	Tree	Bark, seed	Stomach pain, eye pain
13	Mehandi	*Mitragyna parvifolia*	Lythraceae	Shrub	Leaf	Hair dyes, dandruff problems
14	Tulsi	*Ocimum sanctum*	Lamiaceae	Shrub	Leaf, stem, flower	Fever, blackheads
15	Karanj	*Pongamia pinnata(L.)*	Fabaceae	Tree	Root, flower, leave	Skin ulcers, dandruff
16	Arandi	*Ricinus communis*	Euphorbiaceae	Shrub	Seed	Wound healing, fights fungus
17	Sal	*Shorea robusta roxb*	Dipterocarpaceae	Tree	Seed	Digestion, weight loss
18	Jamun	*Syzygium cumini (L.)*	Myrtaceae	Tree	Fruit	Diebetes
19	Giloy	*Tinospora cordifolia*	Menispermaceae	Herb	Stem	Fever, control blood sugar level
20	Imli	*Tamarindus indica*	Fabaceae	Tree	Fruit, flower	Cold, fever

Table 1: Diversity of plants of Jashpur District

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S. No.	Family	Number of species
1	Rutaceae	2
2	Maliaceae	1
3	Anacardiaceae	1
4	Acanthaceae	1
5	Fabaceae	4
6	Solanaceae	1
7	Caricaceae	1
8	Apocynaceae	1
9	Mulberry	1
10	Lythraceae	1
11	Lamiaceae	1
12	Menispermaceae	1
13	Euphorbiaceae	1
14	Dipterocarpaceae	1
15	Myrtaceae	1
16	Zingiberaceae	1

Table 2: Plant species distribution according to their family

S.No.	Habit	Number of plant species	Distribution (%)
1	Herb	04	23
2	Shrub	04	23
3	Tree	12	36
4	Total	20	90

Table 3: Distribution of plants as per their habit

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1. *Azadirachta indica*

2. *Curcuma longa*

3. *Tinospora cordifolia*

4. *Catharanthus roseus*

5. *Capsicum annum*

6. *Carica papaya*

7. *Tamarindus indica*

8. *Ocimum sanctum*

9. *Citrus limonum*

10. *Andrographis paniculata*

11. *Anacardium occidentale*

12. *Shorea robusta*

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Conclusions

Research is happening to check the healthful plants and additional analysis is to be conducted during this field. Diversity should be prohibited at the scale of habitats or ecosystems instead of at the species level. medicinal plants utilized in tribal medication from the Jashpur district are survey and documented consistently. the importance of the plants in ancient medication and also the importance of the distribution of those chemical constituents were mentioned with relevancy the role of those plants in the Jashpur district. As herbs are natural products they're free from side effects, they're relatively safe, eco-friendly, and domestically out there. historically there is are heap of herbs used for the ailments related to totally different seasons. there's a desire to promote them to save lots of the human lives.

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Article 16

Purification of Antifungal Proteins and their Mode of Action on Medically Relevant Pathogens

Kavitha Dhandapani, Karthiga Sivarajan, Ramya Ravindhiran, Ramya Krishnamurthy and Jothi Nayaki Sekar.

Abstract

Invasive fungal infections play a crucial role in mortality and morbidity worldwide, especially in immunocompromised individuals. Many fungal infections in human may lead to various secondary related disorders resulted in severe infections or even a death. Owing to the emergence of new invasive fungal infections, efficient antifungal drugs with targeted action is much needed. Hence, the present study is on evaluating the antagonistic efficacy of *Aspergillus giganteus* and its antifungal protein on pathogenic *Cryptococcus neoformans* and *Candida albicans*. The growth inhibiting ability of *A. giganteus* against pathogenic fungi of *Cryptococcus neoformans* was assayed with dual culture. Various day culture filtrates of *A.giganteus* (5, 7, 9 and 14) were used to study the antagonistic effects against selected pathogens. The antifungal compounds responsible for the antagonistic property was precipitated by TCA/Acetone method. The inhibitory characteristics of purified fractions were also tested on pathogenic *Cryptococcus neoformans* and *Candida albicans*. Among the different day culture filtrates used, the maximum inhibition was seen in the culture filtrates obtained on 7th day of its growth stage. The purified fractions have also exhibited immense antagonism against medically important fungal pathogens. This study proves that the compounds which are responsible for the antagonistic efficacy may provide valuable information in the field of healthcare and medicine.

Keywords *Aspergillus giganteus*, TCA/Acetone precipitation, culture filtrates, antagonism

1. Introduction

Fungal infections have been contemplated asan alarming threat at global level due to the increased mortality and morbidity rate. More than one billion people has been affected by fungal infections annually where 150 million were severely infected with systemic mycoses (Bongomin *et al*., 2017). Antifungal drugs/compounds have representedas a pharmacologically diverse group of medicine that is crucial components within the modern clinical management of mycotic infections. Even the increased growth of antimycotic pharmacology for the past three decades, common invasive and systemic fungal infections still possess a high mortality rate such as approximately 20 to 40%, 50 to 90% and 20 to 70% for *Candida albicans*, *Aspergillus fumigatus* and *Cryptococcus neoformans*, respectively (Mckeny and Zito, 2020). Almost one billion people are suffered with fungal diseases among

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all 1.5 million deaths have been reported in every year (Tudela and Denning, 2017). Ninety percent of entire deaths are a consequence of fungal disease affected by species of the genera such as, aspergillus, candidaand cryptococcus(Huber *et al.,* 2020). The fungal infections are especially affects the immunocompetent or immunocompromised people with HIV/AIDS or autoimmune diseases, and also who are taking anticancer chemotherapy or organ transplantation (Perlin *et al*., 2017). There are numerous fungi as human pathogens present in nature namely *Aspergillus niger, Candida albicans, Cryptococcal neoformans, Aspergillus flavus, Cryptococcus gatti, Pneumocystis jirovecii, Aspergillus fumigatus, Histoblatoma capsulatum, Blastomyces dermatitidis and Penicillium chrysogenum*. They are the predominant human pathogenic organism causes harmful effects at the specific site in the host (Yang *et al*., 2020).

The opportunistic fungal pathogens are major threats to human and animal health and the fungal species are the reason behind deleterious and harmful infections in host. *Cryptococcus neoformans* is a facultative intracellular fungal pathogen and it is addressed as the causative agent for the deleterious disease cryptococcosis. Cryptococcosis disease primarily affects the individual with impaired immunity especially, the person suffered with advanced HIV infection. This pathogen can be seen everywhere around the environment and environmental conditions ranging from the tropical to the temperate. *Cryptococcus neoformans* infection can occur through inhalation and subsequently it forms infection in the lungs. The cryptococcosis infection may be cleared and contained as granuloma, which may disseminate from its initial site, resulting in pneumonia and meningoencephalitis, the latter being uniformly fatal if untreated. Even though there is a more antifungal therapy and chemotherapy available for treating fungal infections nearly more than 650,000 people die by this cryptococcosis infection caused by *Cryptococcus neoformans* (Crawford *et al*., 2020; Beardsley *et al*., 2019).

Candida species are common commensal fungi that colonized in various mucosal surfaces including, oral cavity, gut or vagina. The increasing invasive fungal infection caused by *Candida albicans* is a global phenomenon which is the causative agents for candidiasis (Toth *et al*., 2019). In healthy patients, *Candida albicans* can causes mild superficial mucosal infections with significant morbidity considered as oral thrush and vulvovaginal candidiasis. This Candida infection can cause a higher mortality and morbidity rate, especially found in patients who are admitted in ICUs and suffered with HIV. Hence, much attention should be paid on understanding the basics of their pathobiology, virulence factors, predisposingconditions along with the immune responses of both healthy and immunocompromised individuals (Nobile and Johnson., 2015; Vila *et al*., 2020).

Only a limited number of fungal metabolites have been studied as potential antifungal compounds for specific systemic fungal infections. Hence, discovery of new antifungal compounds for treating fungal infections caused by opportunistic fungal pathogens becomes an emerging area of drug discovery. The pathogenic cell wall and cell membrane

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being act as the primary targets for antifungal compounds (Lozano *et al*., 2018). A vitalizing lead compound for a replacement generation of fungicides is that antifungal protein AFP from *Aspergillus giganteus*. This fungus secretes antifungal protein which has the ability to inhibit the growth of numerous pathogenic filamentous fungi and non-filamentous fungi. This kind of antifungal protein is highly targeting the chitin synthesis proteins/enzymes in order to affect the cell wall integrity and permeability of both plant and human pathogens. It is evident that has the capacity to modulate the composition of cell wall by increasing the expression of target gene such as, α-1, 3-glucan synthase A gene (*agsA*), through the activation of the cell wall integrity pathway (CWIP) and inhibiting chitin synthesis in sensitive fungi. This is not solely due to its fungus-specific mode of action within the micromolar range but also because of its high stability under the favourable environment of temperature at 80°C, pH ranges from 2 to 10 and protease stresses. Eventually, it targets the spore germination, growth of hyphae and metabolite production of opportunistic pathogen (Paege *et al.*, 2019).

The present study has dealt with the analyzing the antagonistic potential of *Aspergillus giganteus* on medically important fungal pathogens such as, *Cryptococcus neoformans* and *Candida albicans*.

2. Materials and methods

2.1. Collection and maintenance of fungal strains:

The fungal strains of *Aspergillus giganteus* (MTCC 8408) obtained from MTCC – IMTECH, Chandigarh to understand its inhibitory potential against fungal pathogens. The pathogenic fungi namely, *Cryptococcus neoformans* and *Candida albicans* were obtained from PSGIMS, Coimbatore. Both the strains were maintained at czapek yeast extract agar for further studies. Our pilot studies have exhibited the antagonistic properties of active metabolites of *Aspergillus giganteus* against various fungal pathogens (Krishnamurthy *et al*., 2020; Ramya and Kavitha, 2021).

2.2. Preparation of culture filtrates of antagonistic fungi

50ml of czapek basal broth was prepared for each 150 ml conical flask and were incubated with 1 ml of inoculum containing 10^8 spores /ml of *A. giganteus* and incubated at 28°C.The culture filtrates were obtained on different days (days 5, 7, 9 and 14) by filtering the media with 8 layers of cheese cloth, sterile cotton wool and then in Whatman No 1 filter paper under sterile conditions. The crude filtrates were centrifuged at 3000 rpm for 15 minutes to obtain mycelia free supernatant. This filtrate was passed through 0.45 µm syringe filter and used for the following experiment. The protein content present in the culture filtrate was estimated by standard Lowry's method.

2.3. Evaluation of antagonistic activity of different day culture filtrates of *Aspergillus giganteus* on clinically important fungal pathogens:

8 ml of the antagonistic culture filtrates were placed in sterile petridishes followed by pouring 32 ml of

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czapek yeast extract agar, so as to make the final concentration of culture filtrate 40%. The culture filtrates were obtained on different days, such as 5, 7, 9 and 14 days from *A. giganteus* culture and each day's filtrates was analysed at 40% concentration. After the culture filtrates impregnated agar solidified, loop full of pathogenic *C. neoformans* and *Candida albicans* obtained from actively growing fungal plates were streaked on the agar. Control plates contained axenic pathogenic fungi. The petridishes were incubated at 28°C for 4 days and radial growth was recorded every day. The percentage radial growth inhibition (R) was calculated. The experiments were performed in triplicates. The inhibitory potential was expressed by following equation:

$$\text{Percentage of inhibition} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

Where,

Control = growth of fungal pathogen

Test = growth of fungal pathogen in the presence of antagonistic fungi.

2.4. Purification of antifungal protein AFP from *Aspergillus giganteus* by TCA/Acetone precipitation method:

The antifungal proteins from *Aspergillus giganteus* were precipitated by TCA/Acetone method to validate its antagonistic property. TCA/Acetone precipitation method is one of the most common methods to purify the protein from crude sample. The acetone used in this procedure should be pre-chilled and stored in a -20°C freezer until needed and kept on ice during entire procedure. 1ml of cell lysate, 8ml of 100% ice-cold acetone and 1ml of different percentage concentration of TCA, 20%, 40%, 60%, 80% and 100% respectively, were taken in the ratio of 1:8:1. It was allowed to precipitate at -20°C for 1hour and centrifuge at 11,500rpm (18,000 ×g) for 15mins at 4°C in a micro centrifuge. Discarded the supernatant, pellet was washed with 1ml of ice-cold acetone (resuspend pellet completely) then centrifuged at 11,500 rpm for 15mins at 4°C again discard the supernatant and repeated this washing to remove all of the residual TCA. Let the pellet was allowed to dry at room temperature for the removal of acetone. The pellet was stored at 20°C for later use or dissolves the protein pellet in the appropriate volume of 2-D rehydration buffer by repeatedly pipetting up and down to break up the pellet. The sample was allowed to sit at room temperature for 1hour followed by vortexing approximately every 10mins. After that the contents were transferred to eppendorf tube and centrifuged at 14,000 rpm for 10mins at room temperature. The supernatant was transferred to a new eppendorf tube and stored at -80°C for further use. The protein content in the precipitated fraction was estimated by Lowry's method (Lowry *et al*., 1951).

2.5.

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2.5. Antagonistic activity of purified sample against fungal pathogens

The czapek yeast extract media was prepared which was poured into a sterile petridishes. 100µl of purified fracction was poured onto the surface of the czapek yeast extract media after that a loop full of pathogenic strain obtained from actively growing fungal plate was streaked on the agar. Control plates contained axenic pathogenic fungus streaked onto the agar plates. The petridishes were incubated at 28°C for 4 days. The percentage radial growth inhibition (R) was calculated. The experiments were performed in triplicates.

3. Results And Discussion

3.1. Inhibitory growth of pathogenic *Cryptococcus neoformans* and *Candida albicans* during co-culture with culture filtrates of *Aspergillus giganteus* on various day treatments

The culture filtrate was prepared and the protein concentration present in the culture filtrate were estimated and the amount of protein was found to be 0.5mg/ml. Table 1 shows the antagonistic activity of different day culture filtrates of *A. giganteus* (5, 7, 9 and 14) on *Cryptococcus neoformans* and *Candida albicans*. The maximum inhibition was noticed in the culture filtrates of *A. giganteus* obtained at 7th day on both the pathogens and the radial inhibition was calculated. It was seen that the culture filtrate obtained from the 7th day of growth showed a maximum inhibition percentage of 90.6±1.8% for *Cryptococcus neoformans* (Figure 3.1) and 93.1±1.8% for *Candida albicans* (Figure 3.2) when compared to the inhibition percentages obtained on other days. The culture filtrates of antagonistic fungi have the ability to combat various fungal infections by inhibiting the growth of fungal pathogens (Djaaboub *et al.*, 2018; Islam *et al*., 2018).

S.No	Day of obtaining culture filtrates from *Aspergillus giganteus*	Percentage inhibition (%)	
		Cryptococcus neoformans	*Candida albicans*
1.	5th day	76.6± 2.1	74.8± 3.1
2.	7th day	90.6± 1.1	93.1± 1.8
3.	9th day	87.6± 1.0	89.7± 1.8
4.	14th day	72.1± 1.0	47.2 ±1.9

Table 3.1: Percentage inhibition (%) of antagonistic culture filtrates treated clinical fungal pathogens

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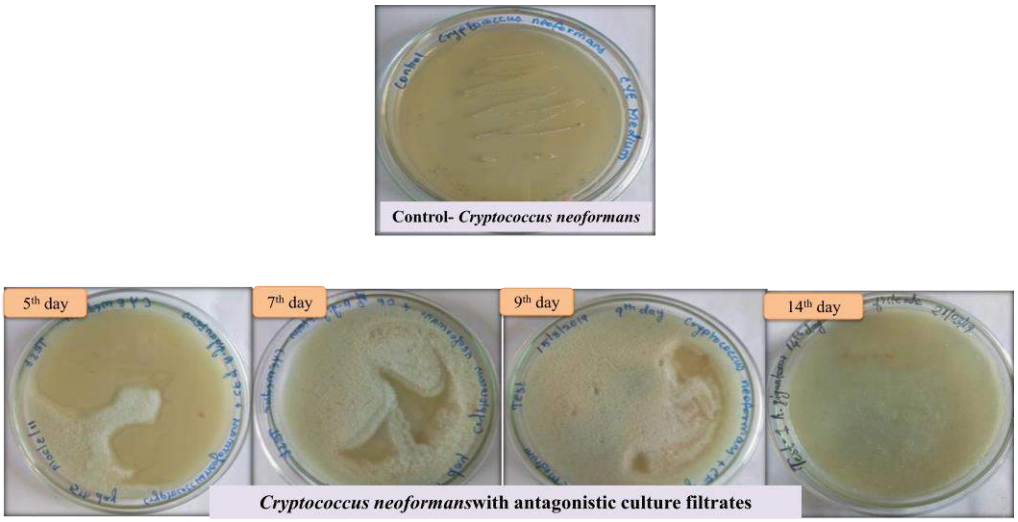

Figure 3.1: Antagonistic potential of *Aspergillus giganteus* at different growth stages against *Cryptococcus neoformans*

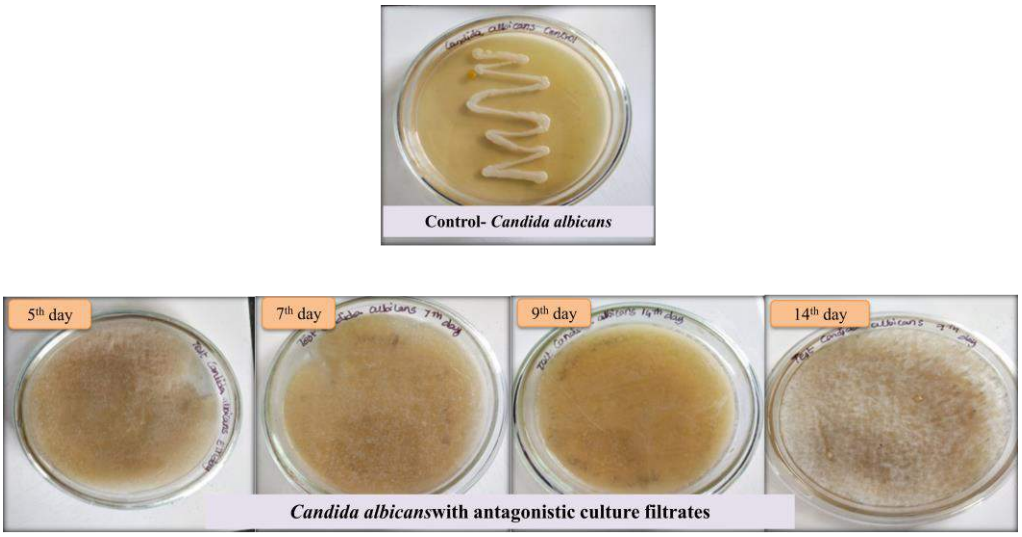

Figure 3.2: Antagonistic potential of *Aspergillus giganteus* at different growth stages against *Candida albicans*

3.2. Purification of antifungal protein AFP from *Aspergillus giganteus* by TCA/Acetone precipitation method

The antifungal protein was precipitatedfrom the culture filtrates obtained from *Aspergillusgiganteus*with the different concentration of TCA, 20%, 40%, 60%, 80% and 100% respectively and acetone. The purified fractions were allowed to dry to remove the residual TCA and acetone and dissolved in Tris-HCl buffer for further use. The protein content of the partially purified compound was estimated by standard Lowry's method. The amount of protein present in the partially purified fractions is represented in Figure 3.3. Various studies have supported the precipitation of antifungal proteins by TCA/Acetone method (Li *et al.*, 2018; Olaniyi and Adebowale, 2017).

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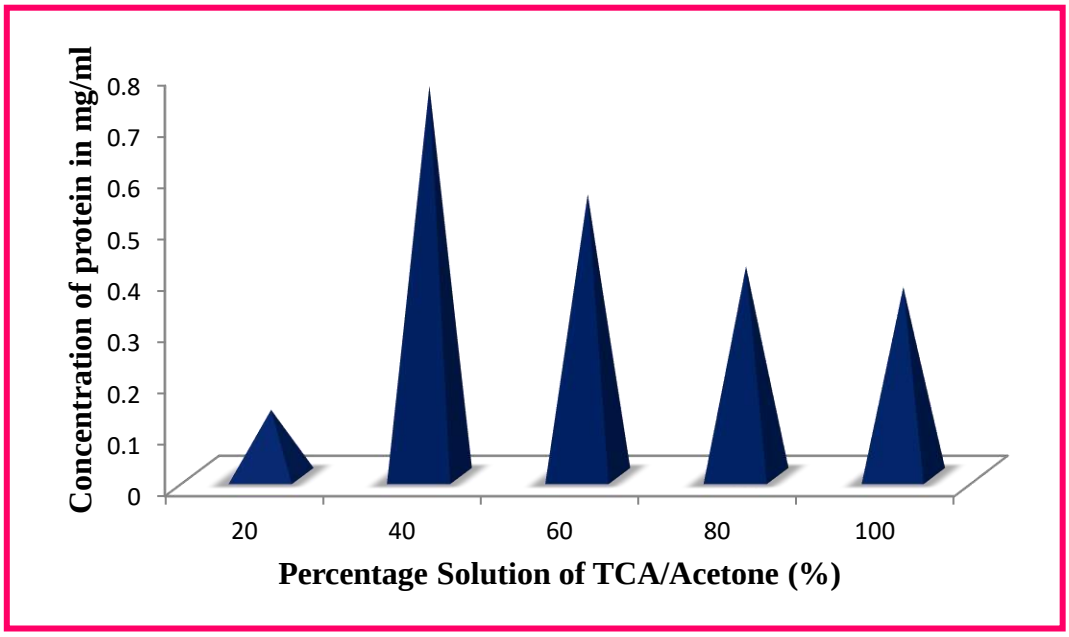

Figure 3.3: Quantification of protein content in the purified fractions using different concentration of TCA/Acetone precipitation method

3.3. Antagonistic activity of purified sample against *Cryptococcus neoformans* and *Candida albicans*

The protein concentration was found to be best in using 40% of TCA/acetone precipitation. Hence, the precipitated protein was taken to prove its antagonistic activity against *Cryptococcus neoformans.* Figure 3.4 explains the inhibitory efficacy of antifungal protein precipitated from *Aspergillus giganteus.* The inhibition percentage was recorded as 88.5% for *Cryptococcus neoformans* and 87.3% for *Candida albicans*.

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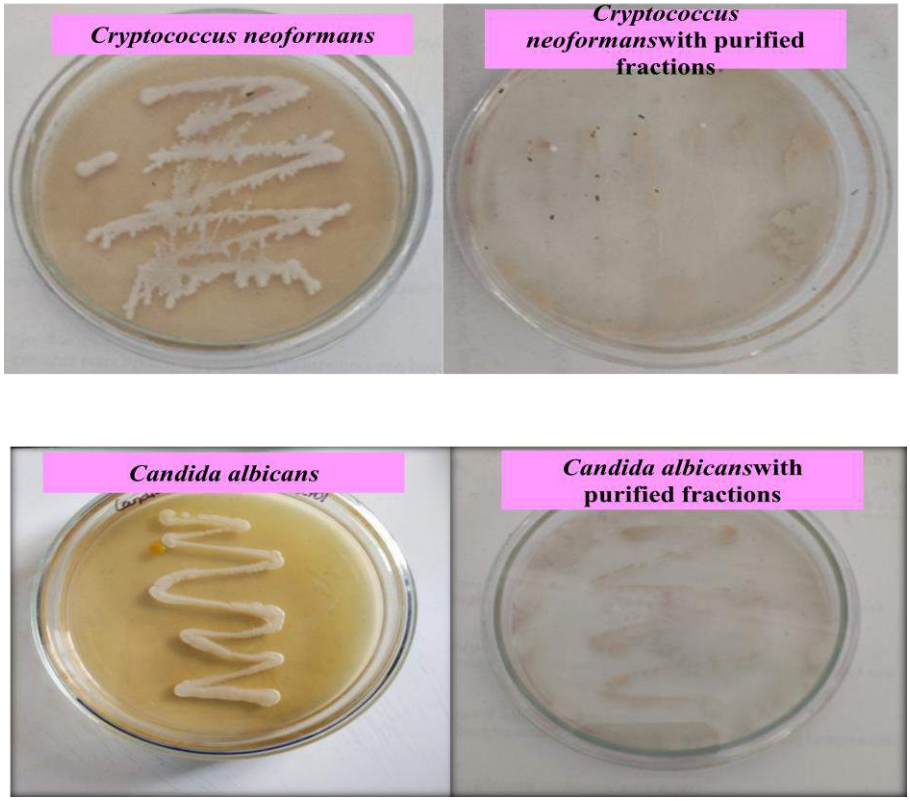

Figure 3.4: Inhibitory efficacy of purified fractions of *Aspergillus giganteus* on pathogenic *Cryptococcus neoformans* and *Candida albicans*

Conclusion

Development of pharmacologically important drug molecules for the prevention and treatment of mycotic infections has gained much attention at global level. The antagonistic efficacy of *Aspergillus giganteus* has been proved on clinically important fungal pathogens namely, *Cryptococcus neoformans* and *Candida albicans*. The purified compounds from the antagonistic fungi showed immense inhibition towards the fungal pathogens. Hence, this study paves the way for the development of novel antifungal drugs in order to inhibit the growth and spreading of pathogens responsible for causing cryptococcosis and candidiasis.

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Article 17

A Study on the Production and Application of Banana Peel Vinegar based Bio-Fertilizer

Balambigai. N and Aswathi. S

Abstract

Eco-friendly products ensure efficiency by using non-harmful materials without sacrificing quality. The objective of this study was to improve the efficiency of bio-fertilizer with the utilization of vinegar and their capacity to improve the yield, suppress pest and disease intensity to maintain sustainable agriculture. The production of vinegar from the banana peel can be of great value by increasing the economic value of the banana. It also provides an avenue to utilize the waste produced from other fruits and vegetables which act as an excellent remedy in waste management. Vinegar is not only a food grade preservative but also used as a base material for the production of organic bio-fertilizer. The acetic acid content present in the vinegar was also tested. The pH of banana peel vinegar is 5.7. Concentration of acetic acid in the undiluted sample of banana peel vinegar is 2.52 mol L^{-1}. Bio-fertilizers produced from biological wastes are used to improve the soil fertility. The pH and nitrogen content of the bio-fertilizers were analysed. The pH of starch-based bio-fertilizer was 4.5. The total nitrogen content of starch-based bio-fertilizer is 0.53. The starch-based bio-fertilizer was applied on flat lentils (*Vignaunguiculata*) for 4 weeks. The number of leaves and shoot length of plants were noted. The starch-based bio-fertilizer acts as an ideal bio-fertilizer as it is cost-efficient and eco-friendly. The importance of this study lies in that it may benefit farmers, farm owners and those who have an interest in agriculture.

Key words Banana peel, Vinegar, Bio-fertilizer, *Vignaunguiculata*and Waste Management.

1. Introduction

The aim of the present work was to use the recyclable and renewable material for the production of green products. The ingredients used in the products are non-toxic and which are not harmful to the environment. The primary objective is to reduce the adverse effects ofthe synthetic products and its consumption and disposal on the environment. This means that the products and services are either eco-friendly or produced in an eco-friendly manner, which does not harm the environment. Vinegar is known as a seasoning or food preserving agent. Vinegar is defined as “a liquid, fit for human consumption, produced from a suitable raw material of agricultural origin. Vinegar is made from sugary or starchy materials by an alcoholic fermentation process followed by acetous fermentation (Okafor, 1987). The objective of this study was to find the possibility of production of vinegar from banana peel through two successive steps such as alcoholic and acetic fermentation. This study also analyses the production and application of bio-fertilizer using the banana peel vinegar.

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Acetic acid is formed in four step reactions involving conversion of starch to sugar by amylases, anaerobic conversion of sugars to ethanol by yeast fermentation, conversion of ethanol to hydrated acetaldehyde, and dehydrogenation to acetic acid by aldehyde dehydrogenase. The last two steps are performed aerobically with the aid of acetic acid forming bacteria. Acetic acid yield improvements can be achieved using high rates of aeration during continuous production. Vinegar bacteria, also called acetic acid bacteria, are members of the genus Acetobacter and characterized by their ability to convert ethyl alcohol (C_2H_5OH) into acetic acid (CH_3COOH) by oxidation as shown below,

$$\mathbf{2C_2H_5OH \rightarrow 2CH_3CHO \rightarrow 2CH_3CO_2H + 2H_2O}$$

Production of vinegar from pineapple peels using Acetobacter species isolated from soil sample andits antimicrobial activity by Sarkar *et al.,* (2012). For this study *Saccharomyces cerevisiae*was isolated from a soil sample and analyses the morphological, biochemical and cultural characteristics of bacteria and fungi. *Saccharomyces cerevisiae* was utilized to produce wine and this was confirmed by performing CO_2 production and iodoform test. The wine produced was inoculated with acetobacter species. It is incubated for 11 days aerobic fermentation at 37℃, it was calculated that 4.60% of vinegar was produced. The antibacterial activity of vinegar was tested against *Escherichia coli, Staphylococcus aureus, Salmonella paratyphi* and *Pseudomonas aeruginosa*. The result of the antibacterial activity of vinegar against Escherichia coli-16mm, *Staphylococcus aureus* -20 mm, *Salmonella paratyphi* -19 mm, and *Pseudomonas aeruginosa*-19mm. Based on this research, they concluded that materials like pineapple peel considered generally as waste can be bio-converted into important value-added materials, thus aiding environmental safety. Study of pineapple peel processing into vinegar was done by Seyram*et al.* (2009). They concluded that a strain of yeast named *Saccharomyces cerevisiae* and acetic bacteria named acetobacter species and acetic bacteria have been isolated from pineapple juice. The production of pineapple vinegar to at least 4.5, brix 5.3%, pH 2.8 requires 23 to 25 days for alcohol and acetic fermentation. From all these results they concluded that post-harvest losses of pineapple fruits may be used to produce vinegar and have a commercial value.

Vinegar production from pineapple wastes was carried out by Arianna Roda*et al.,* (2014). Their research was aimed at completely processing pineapple wastes in to vinegar which may be then used as dressing, food preservative, and disinfectant. Praveena and Estherlydia (2014), carried out studies on phytochemical screening and antioxidant capacities of vinegar made from peel and fruit of pineapple. They produced vinegar from the peel and fruit of pineapple, sugar and starter culture. They assessed the phytochemical properties and antioxidant activity of pineapple peel versus fruit vinegar. They obtained yellow-coloured vinegar from the pineapple fruit mixture. They observed that antioxidant content of peel vinegar (2077mg acetate equivalence/100ml) washigher compared to that of fruit vinegar. They concluded that pineapple peel vinegar can be produced on a large scale and marketed for its therapeutic effects.

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Bio-conversion of papaya peel waste to vinegar using *Acetobacteraceti* carried out by Vikas (2014)and Mridualumesh (2014). This study revealed that dilute acid hydrolysis resulted in the conversion of complex sugars in papaya hydrolysateto simpler fermentable sugars. During the anaerobic fermentation, amylase from yeast initially breaks the starchy residues further into monomeric residues. These residues are utilized for ethanol production. The total ethanol content was found to be 8.11%. Finally the acetic acid fermentation is carried out via the conversion of ethanol to hydrate acetaldehyde and dehydrogenation of acetaldehyde to acetic acid by aldehyde dehydrogenase produced by *Acetobacteraceti*. They concluded that the production of vinegar from papaya peel suggested an adoptable methodology for turning a potential waste in to a commercially important organic acid.

Minh (2014), investigated Fermentation of star fruit juice to produce vinegar using the yeast *Saccharomyces cerevisiae and Acetobacteraceti* bacteria. The researcher used Star fruits collected in the rural area of TraVinh province, Vietnam and observed that at the ethanol concentration of 10%, the acetic acid formation was maximum. At the pH value of 3.5, fermentations were optimum. According to this study, the most common technology used in the vinegar industry is the feed-back method to increase the speed of the acetic acid biological reaction.

Dabija and Hatnean (2014), summarized research concerning the quality of vinegar made from apples, Florina assortment, harvested from Suceava county area. Their study indicated that apple vinegar is obtained through double fermentation. They also found that the quality of vinegar depends on the factors such as the raw material used, the technological process adopted, the technological equipment supplied, acetic bacteria species used etc.

Budak*et al.* (2014), studied functional properties of vinegar. They carried out review of research on these fermented products. It indicated numerousreports of health benefits derived by consumption of vinegar components. They discussed vinegar history, production, varieties, acetic acid bacteria, and functional properties of vinegars. According to these studies, acetic acid is the dominant flavor compound in vinegar. It was also found to have the potent bioactive effects which may benefit human health. The therapeutic properties of vinegar include antibacterial activity, blood pressure reduction, antioxidant activity, reduction in the effects of diabetes and prevention of cardiovascular disease.

1.1. Organic bio-fertilizers

Fertilizers are organic or inorganic, natural or synthetic substances that are added to the soil to enhance plant growth and production. Plants depend on the nutrients in soil to carry out metabolic reactions because the soil contains basic chemicals for plant growth. However, the supply of basic chemicals in the soil to plants is limited. When plants are being harvested, the nutrient content reduces and causes the reduction of the quantity and quality of plants. Fertilizers are applied to replace the chemical materials in soil that are utilized by plants during growth and development. Organic fertilizers are natural fertilizers that are made up of animals, plants and minerals. It can be made up from compost, manure, wood ash and peat moss. Organic fertilizers are crucial in the agricultural sector because they have a positive

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effect on soil without damaging ground water and plants. Organic fertilizers improve soil quality and produce crops with better yield and quality.

1.2. Production of organic bio-fertilizers

The processes in the production of organic fertilizers from food waste include fermentation, solid state fermentation and composting. In the fermentation process, the substrate itself becomes the carbon source and happens in the near absence or absence of free water (Bhargav*et al.,* 2008). However, in solid state fermentation, the process happens in the near absence or absence of free water by employing natural substrate and inert substrate as solid support. Composting is an alternative aerobic treatment of food waste which turns waste materials into humus rich product that nourishes plant and conditions soil (Stabnikova et al., 2005). Fermentation, solid-state fermentation and composting are alternative approaches to manage biological materials. These processes convert food wastesof vegetable and fruit into useful materials such as organic fertilizers for farming.

1.3. Fermentation

Fermentation is an anaerobic process that converts sugar in food waste into acids, alcohol or gases. During the fermentation process, microorganism metabolizes nutrients in food waste and produces carbon dioxide, methane and acids (Bhargav*et al*.,2008). Microorganism such as fungi, bacteria and yeast digest organic compounds in food waste and produce organic acids such as lactic acid, acetic acid and butyric acid. Microorganism uses simple compounds of food source such as sugar, protein and starch in fermentation. The compounds with relatively high C: N ratio and high water content are suitable for fermentation by microorganism. A small amount of water is liberated by microorganism and fermenting materials during fermentation. The suitable temperature for fermentation is 25°C to 37°C and optimum pH is around 4.0 to 5.5. High temperature can kill the microorganism that is used in fermentation because most of the microorganism is mesophilic which grows at 20°C to 45°C. Too acidic or alkaline condition is not suitable because food waste is pasteurized and nutrients are preserved for a long time when the pH drops below 4.2.

1.4. Food waste

The conversion of food waste into organic fertilizers by fermentation, solid state fermentation or composting is widely conducted to reduce the amount of food waste produced daily (Stabnikova*et al.,* 2004). Food waste that is not handled properly can cause contamination of groundwater, emission of toxic gas, emanation of odour and attraction of vermin. The proper decomposition of food waste can cause the reduction of pathogen and odour (Stabnikova*et al.,* 2004). Food waste such as fruits, vegetables, grain, bread and eggshells can be composed and converted into organic fertilizers. Food waste such as red meat and bones can also be composed, but they take longer time to compose. Food waste is widely recycled into organic fertilizer because food waste has special features as a raw compost agent. Food waste contains high energy and is suitable for energy production and waste stabilization (Oladapo*et al.,* 2012). The ratio of carbon to nutrients of organic wastes

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iscrucial during the fermenting and composting process. These processes depend on microorganism that use carbon source to provide energy and nitrogen to build cell proteins. Nitrogen is the most critical nutrient and requires a small amount of phosphorus and other elements. The C: N ratio in the range of 25-27:1 is considered optimum. The low C: N ratio (<25) can cause the loss of nitrogen from compost via ammonia volatilization. However, the high C: N ratio (>40) can cause immobilization of nitrogen in compost and decrease the rate of decomposition. Vegetable and fruit wastes have C: N ratio of ≤27:1 is moderately suitable for fermentation and composting. The main elements in composting food waste are physical and chemical characteristics of the substrate which include the composition and particle size (Oladapo*et al.,* 2012). Food waste has high moisture content and low physical structure as compared to sewage sludge and manure. Food waste is mixed with bulking agents such as yard waste and sawdust that contain high C: N ratio to absorb more moisture and add structure to the mix thus enhancing composting of food waste. Environmental conditions such as temperature and pH affect the degradation of each component of food waste into organic fertilizers. Carbohydrate, protein and cellulose have different optimum temperature, pH and retention times for compositing. Oladapo*et al.* (2012), found that the temperature of composting food waste is in the range of 28°C to 65°C and the pH is 6.3 to 7.1.

1.5. Production of banana peel vinegar

The banana (matooke) peel has about 2% starch adhering to it after peeling; it was hypothesized that this can be further processed by fermentation into a valuable product such as vinegar. The production of vinegar from the matooke waste can be of great value to the country both economically and will also provide an avenue to utilize the vast waste produced in the form of peels(Byarugaba-Bazirake*et.al.,* 2014).

2. Materials and Methods

2.1. Materials required

Fresh banana peels – 250 Grams, sterile knife -1, yeast- 1gram, filter or muslin cloth, water-1½ Litre, sterile container- 1, mother vinegar- 100 cc.

Methodology:

- Fresh healthy matooke were peeled with a sharp stainless steel knife.
- The matooke peels were washed thoroughly in water and drained for 30 minutes.
- The peels were then sliced into pieces of about 3cm thick, and heated at 98℃ in 1 L of water for 20 minutes.
- The boiled mixture was cooled to room temperature filtered using a muslin-cheese cloth.
- The first and second filtrates were then blended and ½ L of water added in a container.

Activation of dry yeast:

Add 1gram of dry yeast in warm water and 1 gram of sucrose and rest it for 10 minutes.After 10 minutes, the yeast will get activated.

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- Add the activated yeast in the same container.
- Close the lid tightly and incubate it for 3 weeks.
- After 3 weeks, add 100cc of mother vinegar to it and incubate for 1 week.
- After 1 week, the banana peel vinegar is ready to use.

2.2. PRODUCTION AND APPLICATION OF STARCH BASED BIO-FERTILIZER:

Rice (*Oryzasativa*) and Sweet potato (*Ipomoea batatas*) are one of the world's most important staple food crops and a major source of starch products used in industrial production (xu*et.al*.,2014).

MATERIALS REQUIRED:

Banana peel vinegar – 20ml, cotton cloth, water-1 Litre, milk – 180 ml, container -1, elastic band, soaken rice water -20 ml.

Figure 1: Vinegar production from banana peel

METHODOLOGY:

- Add banana peel vinegar in a container.
- Rinse rice in water for 5 minutes.
- Fill the jars 2/3 with water and 1/3 with soaken rice waterand cover with a cotton cloth an elastic band.
- The cotton cloth allows for air exchange. Place jars outside, under a box. The box stops UV sunlight rays from killing the bacteria growing in the water.
- Let it be for two days.
- Take fermented rice water and fill 1/10 of it into new glass jar.
- Fill the remaining 9/10 of the glass jar with milk.
- Cover with cotton cloth to allow for air exchange. Place on a shelf with no direct sunlight.
- Let it be for 5-7 days.
- Separate liquid from curds. This liquid is called Lactobacillus.
- Store LAB serum in glass jar with lid tightened. LAB has 6 month shelf life.
- Dilute 1 tablespoon LAB serum with 1 gallon of water and apply to soil/compost and plants generously. Apply as needed.

3. Results and Discussion

3.1. Banana peel vinegar

4. The banana wine vinegar production process took 28 days which complied with the standard ranges of brewed vinegar after complete fermentation. This study therefore, showed that matooke peels can be used as an ideal substrate for the production of good

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quality vinegar. This not only increases the economical and food value of matooke but also provides a way of utilizing banana waste.

DAY-1 **3RDWEEK** **4TH WEEK**

Figure 1: Vinegar production from banana peel

4.1. EVALUATION OF BANANA PEEL VINEGAR

Particulars	pH	Concentration of acetic acid in undiluted sample
Banana peel vinegar	5.7	2.52 mol L^{-1}

Table 1: Evaluation of banana peel

4.2. PRODUCTION AND APPLICATION OF STARCH BASED BIOFERTILIZER:

Figure 2: Banana peel Vinegar based lab serum-Starch based bio-fertilizer

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4.3. Evaluation of starch based bio-fertilizer:

S.No.	Particulars	pH	Total Nitrogen content (Ananlysis report from TNAU)
1	Banana peel vinegar based starch bio-fertilizer	4.5	0.53

Table 2: Evaluation of starch based bio-fertilizer

The combination of 1 tablespoon (strained - no solids) of bio-fertilizer with 1 gallon of water is the perfect combination for healthy and nutritious vegetation. The starch bio-fertilizer acts as an idealbio-fertilizer as itcosts efficient and made out of waste.

4.4. Application and analysis of plant growth

Starch bio-fertilizer was applied on *Vignaunguiculata*and analysed the growth patterns for about 4 weeks. The leaves production and shoot length of plants were noted. Starch bio-fertilizer is applied on flat lentils.

Plants growth parameters	Control plant	Banana peel vinegar based Starch bio-fertilizer
Shoot length (in cm) /plant	**5**	**6.8**
No.of leaves produced /plant	**2**	**2-3**

Table 3: Vinegar based Starch bio-fertilizer- applied on flat lentils(*Vignaunguiculata*) (1ST Week)

CONTROL PLANT

BANANA BASED

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Figure 3: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (1ST Week)

Plants growth parameters	Control plant	Banana peel vinegar based Starch bio-fertilizer
Shoot length (in cm) /plant	9.3	12.7
No.of leaves produced /plant	2-3	3-4

Table 4: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (2nd Week)

CONTROL PLANT **BANANA BASED**

Figure 4: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (2nd Week)

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Plants growth parameters	Control plant	Banana peel vinegar based Starch bio-fertilizer
Shoot length (in cm) /plant	13.9	16
No.of leaves produced /plant	4-5	5-6

Table 5: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (3rd Week)

CONTROL PLANT **BANANA BASED**

Figure 5: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (3rd Week)

Plants growth parameters	Control plant	Banana peel vinegar basedStarch bio-fertilizer
Shoot length (in cm) /plant	17.7	21
No.of leaves produced /plant	6	6-7

Table 6: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (4th Week)

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CONTROL PLANT **BANANA BASED**

Figure 6: Vinegar based Starch bio-fertilizer- applied on flat lentils (*Vignaunguiculata*) (4th Week)

The results of the present study shows that the vinegar produced from banana peel was environment friendly. The raw materials which are considered as waste and easily available and also cost effective. Arianna Roda*et al*., (2014) and Praveena and Estherlydia (2014) and many other produced vinegar from pineapple and papaya peels. The present study also proved the same. Banana peel vinegar is produced by the fermentation of alcohols to acetic acid. The acetic acid value of the vinegar was tested. Even though the vinegar can be used as a preservative, this can also be used for the bio-fertilizer production. These peels are usually considered waste, these peels for the production of vinegars, which leads to organic wastemanagement. This vinegar's acetic acid content was also tested, and it was found eco-friendly.

Fertilizers are usually inorganic in nature; to break that we produced organic bio fertilizer using starch content and vinegar. In the present study starch bio-fertilizer was produced using the organic vinegar. The basic necessities of plants like nitrogen and starch was available in this fertilizer. The pH was 4.5 almost which is considered as healthy for soil. The banana peel vinegar based starch bio-fertilizer had very good nutrient content and helps for the development of shoot length and count of leaves. Starch bio fertilizer was produced using soaken rice water and vinegar. Thesoaken rice water is easily available at home and is eco-friendly. This replaces the chemical content in inorganic fertilizers. The fertilizer was used in small quantities mixed with water and applied to plants in a regular time interval. The growth of the plant was noted and it was found to be effective hence proving the efficiency of the fertilizer. This fertilizer is rich in starch content.

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Conclusion

This study, therefore, showed that banana peels can be used as an ideal substrate for production of good quality vinegar.Fertilizers are usually inorganic in nature; to break that, we produced organic bio-fertilizers using banana peel vinegar. Bio-fertilizers produced from biological wastes are used to improve the soil fertility. The starch bio fertilizer acts as an ideal bio-fertilizer as it costs efficient and made out of waste. The importance of this study lies in that it may benefit farmers, farm owners and those who have an interest in agriculture. It also directs them to the pattern of organic agriculture by replacing the chemical fertilizers with natural fertilizers made from household waste in order to improve soil fertilization as well as balancing soil acidity.

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Article 18

Endemic Dung Beetles (Coleoptera: Scarabaeidae: Scarabaeinae) Associated With A Plantation Belt of South Western Ghats, India

Subha Babu Jayaprakash

Abstract

Dung beetles are an extremely diverse and abundant group of insects that perform many ecological functions. This large quantity of ecological functions arises because of their habit of burying countless food resources such as faeces, carcasses, etc. Biodiversity conservation within the natural reserves is always prioritized, but conservation efforts outside the protected areas like plantations where most human activities take place have been little considered. During the colonial period, cold wet conditions in the Wayanad region of south Western Ghats lead to the replacement of natural forests and the establishment of plantations like tea, coffee etc. But nothing is known about the effect of these developments on the dung beetle community. There are no records on how far the habitat modification might have led to the decline and disappearance of endemic dung beetle species reported earlier by Arrow (1931) in the Western Ghats. So in the present study, dung beetles were collected from a coffee plantation belt of Wayanad, a part of Nilgiri Biosphere Reserve of South Western Ghats using dung baited pitfall traps. Thirty-eight species, belonging to 10 genera and six tribes were collected. Among them, eight species were endemics to the Western Ghats. The high incidence of endemics equal to the forest region highlights the significance of coffee plantations in the Western Ghats.

Keywords Dung beetles, Scarabaeinae, Endemics, Coffee plantation, South Western Ghats.

1. Introduction

Dung beetles are an important biotic component of most terrestrial ecosystems belonging to the subfamily Scarabaeinae, characterized by the use of dung and organic debris at both the adult and larval stages (Hanski and Cambefort, 1991). They are coprophagous insects that consume microorganism rich liquid portion of mammalian dung in the adult stage and use fibrous material to incubate their larvae (Halffter and Matthews, 1966; Halffter and Edmonds, 1982;). They play a key role in both the forest and agricultural ecosystem. Their ecological roles involve, removal of faeces from the environment (Tyndale-Biscoe, 1994), soil fertilization and aeration (Bornemissa and Williams, 1970), recycle nitrogen, organic carbon and other nutrients (Rougon and Rougon, 1991), protect seeds from predation (Estrada & Coates-Estrada, 1991; Feer, 1999; Andresen, 2001), seed dispersal (Anderson, 2006) and reduce egg and larval populations of disease-causing flies, hookworms present in the fresh dung of mammals (Miller, 1954; Hanski, 1991) and serve as food for many birds and mammals (Ratcliffe, 1991).

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Cambefort & Hanski (1991) classified dung beetles into three functional guilds based on their nest building and resources relocation categories namely, tunnelers (paracoprid nesters), dwellers (endocoprid nesters) and rollers (telecoprid nesters). Tunnelers construct underground vertical chambers in close proximity to the dung pat and build their nest using the dung from the pat while rollers form compact dung balls from the dung pats which are rolled off and buried in the ground for feeding and breeding whereas dwellers feed and breed in the dung pats itself (Halffter and Edmonds, 1982; Cambefort and Hanski, 1991; Holter et al.,2002). In Scarabaeinae, dung tunnelling is related with tribes Coprini, Onitini and Onthophagini, dung rolling with tribes Scarabaeini, Gymnopleurini, Sisyphini and Canthonini, and dwelling with tribe Oniticellini (Hanski and Cambefort, 1991b). Based on the diel activity of beetles two major temporal guilds are identified namely, nocturnal and diurnal guild (Janzen, 1983; Cambefort, 1991; Doube, 1991). It is a mechanism to keep away from competition between closely related species or phylogenetically distant groups (Cambefort, 1991).

Tropical rain forests are the functionally significant terrestrial ecosystems supporting more than half of global biodiversity (Myers et al., 2000). The Western Ghats, a biodiversity hotspot in southern India is scattered with plantations that were once tropical rain forests (Dolia et al.,2008) and the forest belt in the region is extremely attenuated and fragmented and is now under maximum destructive pressure (Nair, 1991). The establishment of plantations, especially tea, coffee, and eucalyptus is the major cause of forest deforestation in the Western Ghats (Raman, 2006). Conversion of these ecologically sensitive forests to smaller fragments of variable size and agricultural plantations in Wayanad peaked in the 1980s (Nair, 1991). Conservation of rainforest fragments requires an assessment of lands outside conservation reserves that include secondary forests, habitat fragments, and plantations (Brown and Lugo, 1990; Turner and Corlett, 1996; Daily, 2001). Also, a strong need exists for the documentation of the impact of habitat modification on insects in the south Western Ghats by analysing the faunal assemblages now existing in the modified habitats (Vinod, 2009). But there are no records on how far the habitat modification might have lead to the decline and disappearance of many rare and endemic dung beetle species reported earlier by Arrow (1931) from the Western Ghats (Sabu, 2011). With this outlook, a coffee plantation in the Wayanad region, an ideal representative of the transformed habitat is selected for this work.

2. Materials and Methods

3. Study area

The study was carried out in a coffee plantation at Ambalavayal in the Cherambadi belt of South Wayanad (Kerala) which is a part of the Nilgiri Biosphere Reserve (NBR) of South Western Ghats.

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3.1. Sampling

Beetle specimens were collected by pitfall traps baited with the dung of cow, pig and goat from January to December 2015. Plastic basins were used as pitfall traps, 10cm in diameter and 15cm deep and a mixture of mild detergent (to reduce surface tension and facilitate rapid drowning of the beetles) and salt (to reduce deterioration of the specimens) were used as a preservative (Spector and Ayzama, 2003). The basins were buried with their rim is level with the soil and topped with a plastic plate supported on iron bars to prevent desiccation and flooding. At the top of the basin, two hundred grams of fresh dung was placed on a wire grid as bait. Thirty traps spaced at 50 m intervals between traps were placed to minimize trap interference (Larsen & Forsyth, 2005). The trap contents were collected at 12 h intervals to separate diurnal and nocturnal species because the flight activity of dung beetles differs strongly between day and night (Krell et al.,2003). Beetles were identified to species levels using Arrow (1931) and Balthasar (1963) taxonomic keys and deposited in the insect collections of Tamil Nadu Agricultural University (TNAU), Coimbatore. The species were grouped into functional guilds (dwellers, rollers and tunnelers) following Cambefort & Hanski (1991) and temporal guilds – (nocturnal, diurnal, and generalists) following Krell et al. (2003). Species with >10% abundance and reported all the seasons were treated as major groups.

4. Results

A total of thirty-eight species belonging to 10 genera namely, *Caccobius*, *Catharsius*, *Copris, Paracopris,Tibiodrepanus*, *Ochicanthon, Oniticellus, Onitis, Onthophagus* and *Sisyphus* and six tribes Onthophagini, Coprini, Onitini, Oniticellini, Canthonini and Sisyphini, were captured. Out of these 8 species were endemic species to the Western Ghats, namely *Ochicanthon laetus*, *Ochicanthon tristis*, *Onthophagus andrewesi*, *O. amphicoma*, *O. bronzeus, O.devagiriensis, O. tnai* and *Paracopris davisoni* (Fig.1). *Paracopris davisoni* (17.46%)was the second major species in the assemblage. All endemics accounts for 26.6% of overall abundance. The functional guild of all the endemics was tunnelers expect genus *Ochicanthon,* whichbelongs to the roller guild. The temporal guild of majority endemics was generalists expect *Onthophagus andrewesi* and *O. tnai* that were diurnal species.

4.1. Discussion

The presence of 8 endemics (26.6%) and predominance of *Paracopris davisoni,* an endemic species to the Western Ghats indicates that the study region still supports a sizeable number of endemic dung beetles even after the habitat modifications and highlights the chance of revealing many more endemic species from theregion. Moist forests (Tholpetty) of Wayanad Wildlife Sanctuary recorded eight endemic species (Sabu, 2011) and eleven endemics reported from the deciduous forests of the Wayanad region (Vinod, 2009) highlights the importance of coffee plantations, since it holds comparatively similar endemics to larger forest region.

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Dung beetle studies from many agricultural habitats of south Western Ghats recorded a lesser number of endemics (Vinod, 2009; Latha, 2011; Simi, 2014). Only 5 endemics species were recorded from the other agriculture habitat (mixed crop) of Wayanad (Vinod, 2009) shows the significance of coffee plantations as a region of conservation priority. A similar study from the coffee agricultural habitat of Ranipuram (Kasaragod district in Kerala) which constituted about six endemic species (19% of the total collection) indicated that the habitat modification of the natural landscape into coffee plantations did not lead to the disappearance of endemic species and the endemic species were able to survive in the newly modified environment (Simi, 2014).

Among the eight endemic species recorded, genus *Ochicanthon* was only reported from the moist forest patches earlier (Krikken and Hujibregts, 2007; Latha et al.,2011). The distributional pattern of both *Ochicanthon laetus* and *O. tristis*, in the Indian subcontinent, is notable that they are confined to the moist forests of southwestern and northeastern India (Arrow, 1931; Balthasar, 1963; Paulian, 1980).

The dominance of tunneler functional guild in both the forest and plantation habitats is the usual pattern of dung beetle assemblages in the Western Ghats (Sabu et al., 2006, 2007; Vinod and Sabu, 2007; Vinod, 2009) and across the globe (Cambefort and Walter, 1991; Andresen, 2005). Aggressive and superior competitive nature of tunnelers in utilizing the dung resource most rapidly (Doube, 1991; Krell-Westerwalbesloh et al.,2004) and easier digging the wet and soft soil due to high moisture in coffee plantations soils contributed to their success and dominance in the various habitats (Scholtz et al., 2006). The high abundance of generalists is attributed to the high dung resource availability during day and night and the low abundance of more competitively superior diurnal and nocturnal beetles (Simi, 2014). It is also attributed to the cool and shaded environmental conditions in coffee plantations that lead to uniformity in the habitat conditions enabling better conditions for generalist species during day and night (Subha, 2018).

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Article 19

Screening of Food-Origin Lactic Acid Bacteria and Evaluation of Its Probiotic Properties

Subhashini Ramakrishnan, Jagadeshwari A S, Kathiresh K, Kiruthikaa L, Jincy Johnson, Kalaiselvi M., Shanmugapriya M and Sreebala G

Abstract

Nutritious foods are rich for its essential nutrients which welfares human health. The food source serves as a rich biodiversity of both beneficial and pathogenic microorganisms. Identification of pathogenic microorganisms from such sources improvisesthe treatment for food borne diseasesby developing drugs or antibioticsto combat against the harmful organisms. However identifying the useful microflora from food sources are important for food industries to come out with nutritional products with good qualities and flavors. Lactic acid bacteria (LAB) are heterogenous group of microorganisms which are mainly used in the food industries to produce functional products. The present study is to isolate, characterise and identify such LAB from fermented and non-fermented products and analysing its probiotic characteristics makes it possible use in manufacturingfood based products.

Keywords Milk, Curd, Probiotics, Lactic Acid Bacteria, *Lactobacillus plantarum*

1. Introduction

Microorganisms are frequently used in fermentation for influencing the quality and functionality of foods by improving the texture, flavor, suppressing the food spoiling pathogens and prolonging the shelf-life. Only few strains are safe and used for producing such products.Safer strains have been also used as probioticswhich improves the nutritional and gut flora balance. Nevertheless, it is also used as a starter culture for the production of fermented products in food and beverage industries.Probiotic products enhance immunity and increase the ability to survive against food related pathogens.Probiotics have been isolated from fermented and non-fermented products.The present research focused on isolating strains from both fermented and non-fermented samples. Identifying the efficientspecies or strains with probiotic characteristicsfacilitatethe better understanding to produce desirable products.

2. Materials and Methods

2.1. Fermented and Non-fermented Samples

Fresh raw milk of cow and curd were collected in a separate vial from different farms of Coimbatore district.Thesamplewas transported to the laboratory in an ice box within 2 hand stored at -20 °C for further studies.

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2.2. Microbiological Analysis and Isolation of LAB

10 mL of each sample was homogenized with 90 mL of sterile sodium chloride broth. The obtained stock solution was used to serially dilute the samples up to 10^{-7} dilutions by incorporating 1 mL of the sample into 9 mL of the diluent.1 mL of the appropriate dilutions was spreaded on De Man, Rogasa and Sharpe (MRS) agar plate and incubated at 37ºC for 48 hours.The culture was selected randomly and streaked in the respective media repeatedlyuntil the pure culture is obtained. The isolated pure colonies were maintained at 4 ºC and revived every month. For the longer storage, culture was maintained in the glycerol stock and stored at -80 ºC.

2.3. Morphological, Physiological and Biochemical Characterisation

Both microscopic and macroscopic features of each isolate were studied for their morphology, colour, elevation and opacity.All isolates were screened using Gram's staining and catalase test and further analyseditstemperature tolerance, NaCl tolerance, fermentative capacity, motility analysis andhydrolysis of esculin. The biochemical characterization of the selected isolates were confirmed using IMViCtests, hydrolysis of gelatin, starch and casein, production of hydrogen sulfide, nitratereduction and oxidase test.

(i) Temperature Tolerance

Isolates were grown in the MRS broth and incubated at different temperatures (10 °C, 37°C and 42°C). After 48 h of incubation, the growth was measured by reading the optical density (OD) values at 600nm.

(ii) Sodium Chloride (NaCl) Tolerance

Freshly prepared 10 mL of the broth was added with different concentrations of NaCl (2%, 4% and 6.5%). To the above sterilized broth, 0.1 mL of overnight culture was inoculated and incubated at 37 °C. After 48 h of incubation, the growth was determined at 600 nm using spectrophotometer.

(iii) Carbohydrate Fermentation Test

Fermentation medium was prepared and added 0.1g of each sugar substrate separately (Sucrose, Lactose, Galactose, Fructose, Dextrose) in the test tube and sterilizedit for 15 min at 121°C. Then inoculated with the single colony of the isolate under study and incubated at 37 °C for 48-72 hrs. Durham tubes were also placed onto the medium to trap the released carbon dioxide. The positive reaction of isolates is indicated by the colour change of the medium. The fermented media which turn green colour in to yellow (indicates acidic) or bluecolour(indicates alkaline).

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(iv) Motility Test

Motility test was performed (Barrow and Felthman, 1993) by using the broth culture in the cavity slide and examined under microscope.

(v) Esculin hydrolysis

The ability of an organism to hydrolyze esculin as carbon source is confirmed by the blackening of the medium. Bile esculin broth was prepared, sterilized, inoculated with 18-24h culture and incubated at 37 °C.

Probiotic Characterization of Isolates

(i)Acid tolerance

10mL of MRS broth with different acidic pH values (1, 2, 3, 4, 5, 6) was maintained with HCl and NaOH. 0.1 mL of the overnight culture was inoculated into the test tubes and incubated at 37°C. Next, the growth was observed for every three hours intervals (3h, 6h) by Spectrophotometric method and its optical density (OD) value was recorded at 600nm.

(ii) Bile Salt Tolerance

Freshly prepared 10 mL of MRS broth was added with different concentration of bile salt (0.5%, 1% and 2%). From the overnight culture, 0.1 mL was inoculated into the bile salt containing tubes and the suspension was incubated at 37°C. The growth was observed for every 3 h intervals (0h, 3h, 6h) of incubation and turbidity was measured at 600nm.

(iii) Cell Surface Hydrophobicity

The cells were washed twice with Phosphate Buffer Saline (PBS) and the OD was measured at 600 nm. 1 mL of the bacterial suspension was added with 1 mL of the xylene solvent. The mixture was then vortex for 1 min and left to stand 15 min to separate into two phases at roomtemperature and aqueous phase was determined using spectrophotometer. Percentage hydrophobicity was calculated according to this formula $(1\text{-}A_{after}/A_{before}) \times 100$.

(iv) Auto aggregation and co-aggregation assays

The selected strains and the four test organisms were cultured in the respective medium. The cells were harvested by centrifugation at 5000rpm for 15min and then washed twice and suspended in PBS. Cell suspension (4 mL) was vortexed and auto aggregation was measured at 600 nm at 0h, 1h, 2 h and 3h. Auto aggregation percentage was expressed as $(1\text{-}A_t/A_0) \times 100$, where A_t represents the absorbance at time t=1h, 2h, or 3h and $A_0$is the absorbance at t=0. The method of co-aggregation experiments is same as auto aggregation assay. Equal volumes of each cell suspension of the selected strains and the test organism were vortexedfor about 15sec. The suspension is incubatedat 25°C and absorbance is measured at different time intervals.

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(v) Antibiotic Activity

Antibiotic susceptibility of the specific isolates was assessed against Kanamycin, Gentamicin, Tetracycline and Ampicillin using disc diffusion method. The overnight culture was prepared in the nutrient broth and incubated at 37°C for 24 h. The fresh culture wasthen swabbed onto theMuller Hinton Agar (MHA) plates and placed different antibiotics on the surface of the media. All plates were visualized for the zone of inhibition after the incubation at 37 °C for 24 h(Venkatesan*et al*., 2012).

(vi) Antimicrobial Activity

The selectedisolates were assayed for its antimicrobial activity against a range of common food pathogenssuch as *Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, and Listeria monocytogenes.* Sterile blotting paper discs were dipped inthe culture broth and then placed on to the solidified MHA plates which are seeded with the test pathogens. The diffused plates were then incubated at37°C for 24h. Niacin was used as the standard control(Tambekarand Bhutada, 2010).

(vii) Hemolysis test

The overnight culture was transferred to the blood agar platesupplemented with 5 % sheep's blood and incubated the plate at 37ºC. The incubated plates were observed for the type of hemolysis. The partial hydrolysis of red blood cells (RBC) and the production of green zone represent α-hemolysis; the total hydrolysis of RBC and the production of clear zone around the bacterial colony representsβ-hemolytic. No evident change in the medium constitutes γ-hemolysis (Ray *et al*., 2004)

2.4. Molecular Identification of the Isolate

The isolate possessing the probiotic properties wereamplified and sequenced. The obtained forward and reverse sequences were submitted to the blastn program to identify the organism.

3. Results and Discussion

3.1. Morphological and Physiological Characteristics

Fermentation is one of the oldest forms of preserving foods. Literature survey revealed thatmany research reported LAB from different fermented sources. Comparativelyless number of LAB isolates was identified from non-fermented sources.This research focused on isolating, identifying the LAB from both fermented and non-fermented sources. Four samples from each fermented (curd) and non-fermented (milk) samples resulted with fourteen isolates (six from fermented and eight from non-fermented). All isolates were selected based on their morphological characteristics (named as C1 to C6 obtained from curd and M1 to M8 obtained from milk).These isolates were screened using Gram's staining and catalase test revealed that all are Gram positive and only seven isolates (four from non-fermented sample and three from fermented sample)are catalase negative (Table 1).

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Isolates	Grams reaction	Catalase test
M1	+	+
M2	+	-
M3	+	+
M4	+	-
M5	+	-
M6	+	+
M7	+	-
M8	+	+
C1	+	-
C2	+	+
C3	+	-
C4	+	-
C5	+	+
C6	+	+

+ indicates positive and – indicates negative

Table 1: Gram's Staining and Catalase Test

Carbohydrate fermentation test was performed to investigate the ability of isolates to ferment different sugars (sucrose, lactose, Galactose, fructose and dextrose).Sucroseis fermented by M2, M5 and C1 isolates whereas lactose is fermented by M5 and C3 isolates only. Galactose was used as a substrate by four isolates namely M2, M5, C1 and C3. Likewise, fructose was fermented by all isolates except M7 and C4.Also, isolatesM7 and C3 lacked its ability to ferment using dextrose. All the screened isolates hydrolysed esculin and are non-motile. The biochemical identification of the selective isolates is given in the table 2.

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Biochemical tests	M2	M4	M5	M7	C1	C3	C4
Indole Production Test	+	-	+	+	+	-	+
Methyl Red Test	+	+	-	+	-	-	+
Voges-Proskauer Test	+	-	+	-	+	-	-
Citrate Utilization Test	-	+	+	-	+	-	+
Catalase Test	-	+	-	-	+	-	-
Oxidase Test	+	-	+	+	-	-	+
H2S Production Test	-	-	+	-	+	-	-
Nitrate Reduction Test	+	-	+	+	+	+	+
Starch Hydrolysis Test	-	+	-	-	-	-	+
Lipase Test	-	+	+	-	-	-	+
Casein Hydrolysis Test	-	-	+	-	-	-	-
Gelatin Hydrolysis Test	-	-	-	-	-	-	-

Table 2: Biochemical Characterisation of the isolates

3.2.Probiotic Properties of Catalase Negative Isolates

Probiotics are live non-pathogenic microorganisms which improve the health of the host andalso serve as alternative to antibiotics. The catalase negative isolates were analysed for its ability to grow at both maximum and minimum temperature, an essential factor of probiotic to show their effectiveness. All the seven isolates survived at three different temperatures tested such as 10 ºC, 37 ºC and 42 ºC and the highest growth rate was showed by M5 isolate at 37 °C and 42 °C (Graph1).

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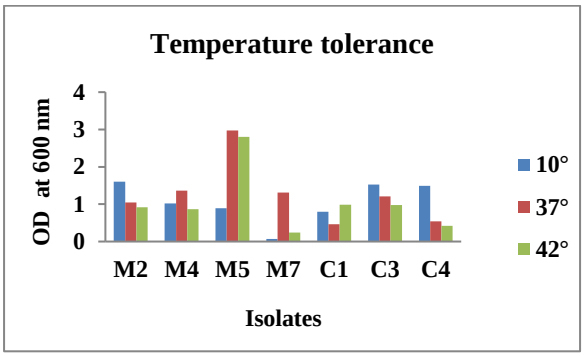
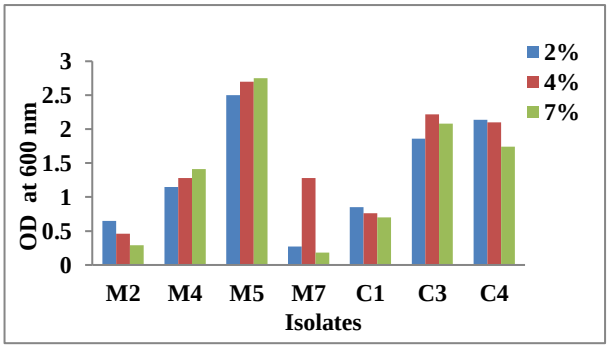


Graph 1: Temperature Tolerance Analysis

Sodium chloride (NaCl) is an inhibitory substance for certain types of bacteria; the probiotic organisms should withstand high salt concentration in the human gut. The isolate M5 grown well on all three different concentrations tested such as 2, 4 and 6.5 % (Graph 2).



Graph 2: NaCl Tolerance Analysis

Acid tolerance test determines the level of tolerance of probiotics at different acidic pH levels while passing through stomach. All isolates obtained from both milk and curd indicated the possibility to survive high level at low acidic pH up to 6 h. The physiological concentration of the digestive fluid salts within the intestine is anywhere between 0.2 and 2 %.The bile salt tolerance analysis showed that M5 isolate from milk and C4 from curd survived better at all the tested concentration of the salt. Cell surface hydrophobicity finds out whether the isolateis hydrophobic or hydrophilic towards the water content in the stomach. All isolates (except M7) revealed thehydrophilic nature was evidenced by its highest growth after adding xylene.

Auto-aggregation and co-aggregation are the other indicative parameters which determinethe capacity of the cells aggregatingeither with the same species or the different species, the adherence nature and molecules involved in mediating the adherence.In the

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present study, both the assay indicated that MH5 isolate showed the potential higher adherencethan the other isolates but lower than the range specified byAlkalbani*et al*., (2019).

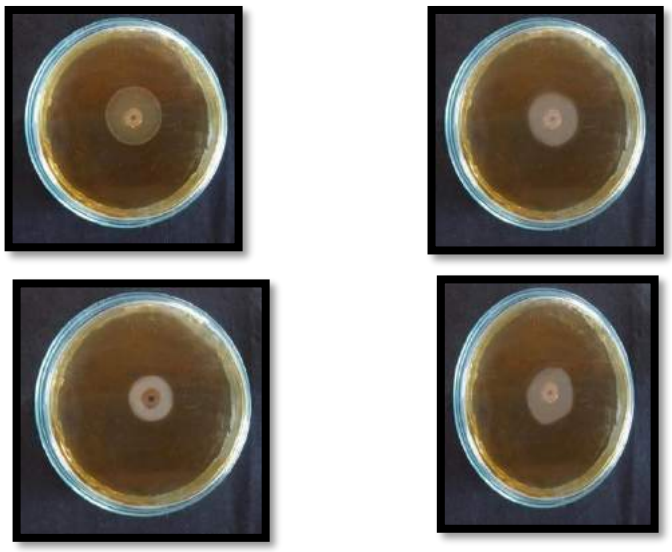

Fig.1: M5 against *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermis*, *Escherichia coli*

Multi-strain or multi-species probiotics have improved functionality when compared to single strain. However, special attention should be given to avoid combination of probiotic strains showing inhibitory properties. Probiotics are needed to be strain specific, condition specific and dose specific. Keeping in this mind, analysed another important feature of probiotic culture which is its ability to kill pathogens. Each isolate was checked for its antimicrobial activity against some common food borne pathogens infecting the gastro intestinal tract are *Listeria monocytogenes*, *Staphylococcus aureus*, *Staphylococcus epidermis* and *Escherichia coli*.The outstanding result attained in the present study is that all the studied pathogens were sensitive only to MH5 isolate(Fig 1). Antibiotic susceptibility pattern of the selected isolates against the four common antibiotics such as Kanamycin, Gentamycin, Tetracycline and Ampicillin tested using disc diffusion method revealed that isolates M5 and C1 were resistant was supported by zone of clearance lesser than <15 mm (Table 3).

Antibiotics	**M2**	**M5**	**M4**	**M7**	**C1**	**C3**	**C4**
Kanamycin	**S**	**R**	**S**	**S**	**S**	**R**	**S**
Gentamycin	**R**	**R**	**R**	**S**	**S**	**R**	**R**
Tetracycline	**S**	**R**	**S**	**S**	**S**	**R**	**R**
Ampicillin	**R**	**R**	**R**	**S**	**S**	**R**	**R**

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S: Sensitive; R: Resistant

Table 3: Antibiotic activity for selected isolates

Haemolysis test was performed to identify the pathogenic or non-pathogenic nature of the M5 isolate showed that the isolate constitutes γ-hemolysis (Fig. 2) as specified by Ray *et al*., (2004). The isolate MH5 has been submitted for molecular sequencing and confirmed it as *Lactobacillus plantarum*, a healthy probiotic strain which supports the digestive system and encourages healthy immune function.

Fig.2: Hemolysis of the M5 isolate

The simplest way of preserving food can be achieved by lactic acid fermentation. The lactic acid bacteria convert carbohydrate to lactic acid. Many such fermenting organisms are identified from human body, dairy foods, non-dairy foods, fermented foods, non-fermented foods, fresh juices, etc.The presence ofsuchbacterium characterises aroma, flavour, texture of food, decreases the spoilage of food by pathogenic organisms and increases the shelf-life of the product.

Azizi*et al*., (2017) stated that LAB strains isolated from dairy and fermented sources may contributethe safety and quality of foods. Amongst, the member from the genus *Lactobacillus* is the first bacteria described as probiotics. The most common probiotic bacteria *Lactobacillusplantarum*is widespread in the environment and have been isolated from different sources such as food containing no milk or milk constituents (Göran, 2001),from fermented products of animal (Azizi*et al*., 2017) and plant origin (Parente*et al*., 2010), from fresh juices (Pairat and Sudthidol, 2016).These strains possess high ability to survive in gastrointestinal tract and regarded as safe strain by World Health Organization (WHO) and FAO (Food Drug Administration).

Gram-positive and catalase negative LAB strains can be isolated from fermented milk samples using MRS agar (Carmen*et al*., 2006).Sedighi-Khavidak*et al*., (2016) isolated seven LAB from different sources where four isolates were identified as *Pediococcusacidilactici*and three as *Lactobacillus plantarum*. Also, the isolated *L. plantarum* from breast milkshowed high inhibitory activity against the aflatoxin producing food spoiling microorganisms. *L. plantarum* isolated from fermented drink was resistance to a low acidic

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pH, 3 % bile salt concentration and also possessed antimicrobial activity against many food borne pathogens (Oluwajoba*et al*., 2012).

In the present study, the identified *Lactobacillus plantarum*showed higher antimicrobial activity against the indicator organisms which is in agreement with the study of Sankar*et al*., (2012). LAB species produces natural food preservatives such as hydrogen peroxide, organic acids, bacteriocins, etc. *L. plantarum*producing bacteriocin was isolated from human breast milk(Shruti and Nitin2019).Bacteriocin producing LAB plays important role inthe digestive tract as it prevents the growth of pathogenic microorganisms. Identifying antimicrobial compounds producing LAB strains promotes the health benefit products(Azizi*et al*., 2017).Changkun*et al* (2017) evaluated the influence of *L. plantarum* on yogurt fermentation which would result in the development of functional foods by dairy industries. Here, the identified *L. plantarum*from raw milk is catalase negative, Gram-positive, aerobic or facultative aerobic, resistant to acidic conditions, produces hydrogen peroxide in addition to its other biochemical characteristics and its molecular characteristics can be explored further for its useas a starter culture for producing commercial fermented products.The *L. plantarum* strain KACC92189 isolated from natural fermented meat is been used as a probiotic starter culture in preparing fermented sausages with improved quality (Hoa*et al*., 2018).

Conclusion

Milk is the nature mother's gift where it affords enormous nutritional benefits. This is evident by the level of lactic acid, pH, protein content, number of LAB. Most fermented dairy products are made from cow's milk.Such functional foods possess superior health benefits than conventional foods. Here, the identified *L. plantarum* is catalase negative, non-motile, aerobic or facultative aerobic, resistant to acidic conditions and bile salt concentrations, produces lactic acid which can be explored further to use it as probiotic starter culture as it satisfied most of the probiotic properties.

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Article 20

Effects of climatic changes on malaria transmission in Delhi, India

Abhishek Gupta and Alka Rani

Abstract

Among the insects, mosquito plays a significant role in transmission of various diseases like Malaria, Dengue, Zika fever, Chikungunya, Yellow fever, Japanese encephalitis, Lymphatic filariasis etc. Transmission of malaria is influenced by various climatic factors like temperature, wind, rainfall, and humidity as reported by several authors. Climate change in Delhi with construction activities and its influence on mosquito borne diseases like malaria entails attention. Thus, the objective of present study was to explore the effects of climatic changes and its impact on malaria in Delhi, India. Malaria vectors of this region like *An.stephensi* and *An.culicifacies* were also studied for their dynamics with changing climatic factors. The data for malaria in urban and rural areas of Delhi, India from 2014 to 2019 was analysed. Rainfall has declined in last few years hence breeding of *An.culicifacies* has decreased whereas breeding of *An.stephensi* was not much influenced. Humidity supports both the vectors. Low rainfall has decreased the abundance of *An.culicifacies* whereas availability of artificial breeding sites has increased abundance of *An.stephensi* in all areas of Delhi. More abundance of urban vector has resulted in more malaria cases in urban areas. The maximum temperature has higher influence on malaria as compared to minimum and average temperature. Hence, both the climatic factors studied influence malaria by influencing dynamics of malaria vectors.

Keywords Climatic changes, malaria, mosquito, temperature, vector

1. Introduction

Malaria is a vector borne disease caused by female *Anopheles* mosquito. Female Anopheles when bites transmit malaria to the host (WHO, 2019). Urbanization, a global phenomenon has impacted vector borne diseases like malaria by influencing host (e.g. human), mosquito vector (*Anopheles* in case of malaria) and parasite (e.g. *Plasmodium* species in case of malaria) (Hay *et al*., 2005; Qi *et al*., 2012; Antonio-Nkondjio *et al*., 2005). It causes climate change, increase in population density via population growth, urban settlements, deforestation or increase or decrease in flora and fauna (McKinney, 2002, 2008). Climate factors like rainfall, humidity, temperature and wind speed influence life cycle of mosquito like *Anopheles* and hence the disease caused by them. For example, temperature increase from 32°C to 39°C has reported to cause high mortality among mosquitoes (Dhiman *et al*., 2010).

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Delhi NCR is a rapidly urbanizing area with varied climate. Delhi has an average temperature 25.2 °C and average rainfall 692mm. The driest month is April and most precipitation falls in August. Ghaziabad has warm and temperate climate. The average annual temperature in Ghaziabad district is 25.1 °C and precipitation is 810 mm annually (climate.org). Delhi was endemic for malaria till 2000, with approximately 5000 annual cases. After 2000s number of cases has come down (NVBDCP Report). Ghaziabad has similar situation of malaria with more than 1000 cases till 2005 which came down to around 200 in 2016 (Ghaziabad PHC). This study will examine how change in climate influence malaria vectors in Delhi NCR region. This will help in knowing their status in urbanizing cities with changing climate.

2. Methodology

This study was done in Delhi and Ghaziabad region. Data on malaria cases from clinics in Delhi was obtained for the period of 2016 from CDC. Malaria cases data for 2015 was obtained from Ghaziabad health centre. Indoor resting *Anopheles* mosquitos were collected from Ghaziabad and Delhi from randomly selected areas. Three weather variables were used here i.e. Rainfall, Temperature (Maximum, Minimum, Average) and humidity. Monthly Rainfall data was obtained from Indian Meteorological department for Delhi and Ghaziabad in 2015 and 2016 respectively.

Temperature and humidity data was extracted from World Weather online for each day and converted to month data for Delhi (2016) and Ghaziabad (2015). Resting Adult *Anopheles* was collected in each season i.e. pre-monsoon, monsoon and post monsoon from randomly selected 5-7 houses (Table 1). Mosquitoes were then brought to laboartoty for identification to the genus and species level (Nagpal and Kalra, 1997). Pearson correlation analysis was conducted to find the relationship between meteorological variables like maximum temperature, minimum temperature, average temperature, average rainfall and humidity with malaria vector (*An.culicifacies* and *An.stephensi)* abundance.

3. Results

About 1269 adult *Anopheles*, of which 608 were vectors of malaria collected from human dwellings and cattle-sheds. Number of *Anopheles* vector found in Delhi were 400 while in Ghaziabad 208. *An.culicifacies* has lower percentage in both Delhi and Ghaziabad with 32.75 %and 26.44 % respectively. Ghaziabad had 7.27 %, 23.64 % and 69.1 % of *An.culicifacies* in urban, peri-urban and rural respectively. *An.stephensi* in Ghaziabad had 50.98%, 34.64 % and 16.99% in urban, peri-urban and rural respectively. Delhi had 69.46%, 12.21 % and 18.32 % of *An.culicifacies* in urban, peri-urban and rural respectively. *An.stephensi* in Delhi had 55.76 %, 21.93 % and 22.3 % in urban, peri-urban and rural respectively.

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Collection Area	Months-year	Rural		Peri-urban		Urban	
		C	S	C	S	C	S
Ghaziabad	Jan-15	11	6	2	17	1	24
	May-15	7	4	1	12	1	19
	Aug-15	20	16	10	24	1	35
Delhi	Apr-16	2	11	1	16	0	22
	Jun-16	18	12	4	8	11	48
	Sep-16	42	21	6	19	10	35
	Oct-16	29	60	5	59	24	150

Table 1 Anopheles (malaria) vector collection from urban, rural and peri-urban are of study sites (Ghaziabad-2015, Delhi-2016) (C=*An.culicifacies*, S=*An.stephensi*)

Month-wise analysis of malaria cases in Delhi region revealed that malaria cases started increasing from the month of March and reached peak in July. Most of the cases were from *Plasmodium vivax. Plasmodium falciparum* has number of cases started rising in August and remained till November (Figure 1). Similarly, Ghaziabad has malaria cases started increasing in March but reached peak in October (Figure 2). Malaria cases correspond with humidity in Delhi while in Ghaziabad both humidity and rainfall influence malaria cases in Ghaziabad (Figure 1 and 2).

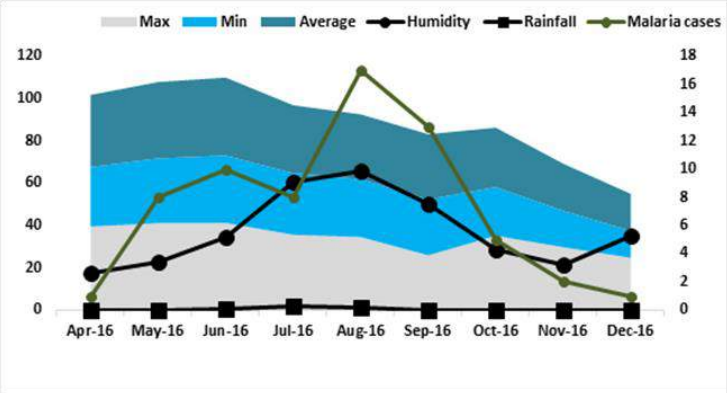

Figure 1 Relationship of climate factors with malaria cases in Delhi during 2016

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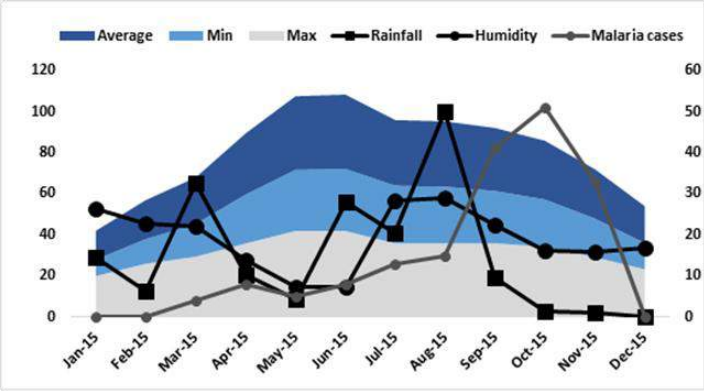

Figure 2 Relationship of climate factors with malaria cases in Ghaziabad during 2015

Pearson correlation analysis as displayed in figure 3 showed the association of climate factors with Malaria cases and *Anopheles* vectors. R values clearly showed all factors i.e. humidity(r=0.76), rainfall (r=0.56) and temperature (min=0.56 and av=0.47) influence malaria cases in Delhi region positively. Ghaziabad has less influence of climatic factors although temperature (min=0.33, av=0.30, and max=0.27) influence positively, rainfall (-0.23) negatively and humidity has no influence (r=0).

Climatic variables influence malaria vectors *An.culicifacies* and *An.stephensi* differently. Temperature effect *An.culicifacies* negatively in Delhi but positively in Ghaziabad. Rainfall has more impact on *An.culicifacies* as it provides breeding sites to this vector and humidity supports it. *An.stephensi* is positively impacted by all the factors except maximum temperature. Delhi has less influence of rainfall compared to Ghaziabad while humidity supports this vector in both the regions (Figure 4). The r values for the all the climatic factors are presented in Figure 3 and 4.

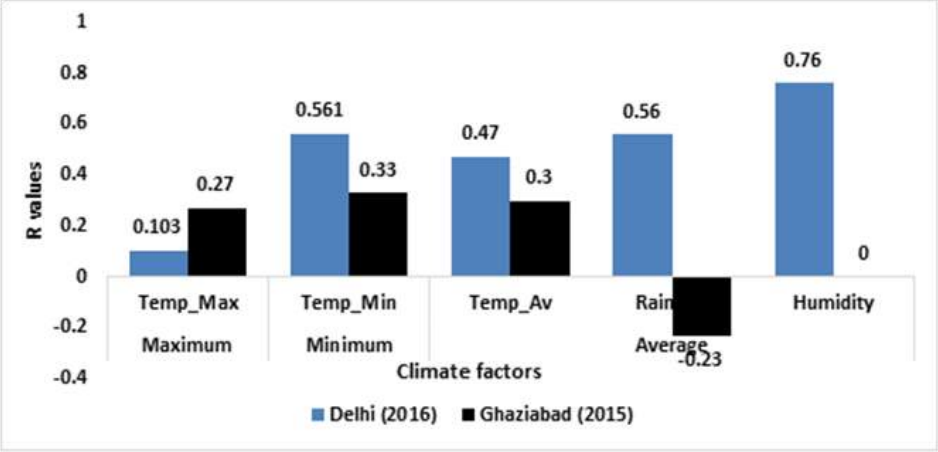

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Figure 3 Association of malaria cases with climate factors using Pearson's correlation coefficient (r)

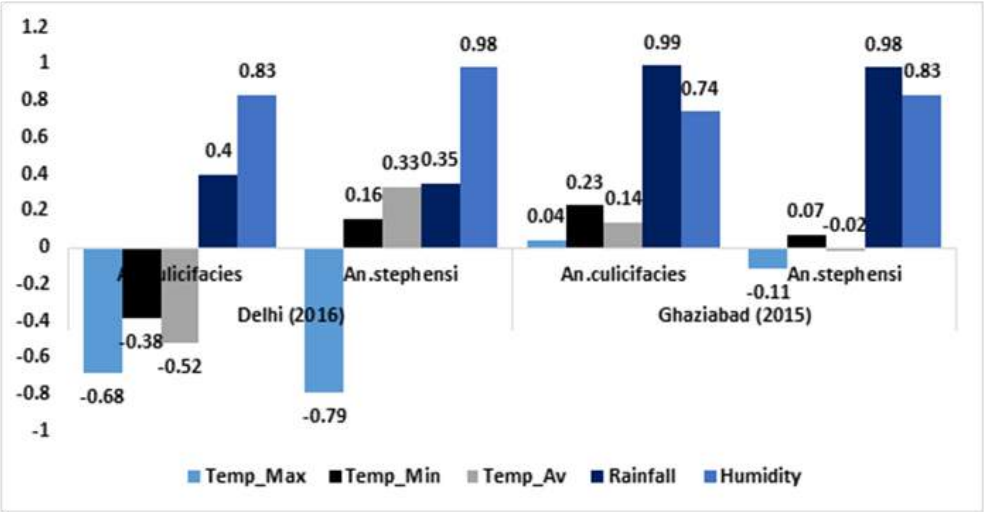

Figure 4 Association of Malaria vectors with climate factors using Pearson's correlation coefficient (r)

4. Discussion

The present study showed influence of climate change on malaria. Climatic factors like temperature, humidity and rainfall affects malaria transmission in various ways. Urbanization has caused increase in temperature and decrease in rainfall in cities. It has also influenced breeding of mosquito vectors and hence abundance (Dash *et al*., 2013). Delhi has urbanization since 1990s while Ghaziabad is experiencing urbanization since 2005 when construction boom started due to population accumulation. *An.culicifacies* and *An.stephensi* are the prevalent malaria vectors in both the study areas. *An.culicifacies* breeds in pits, pools, rivers, ponds, canals and other natural sites while *An.stephensi* breeds in containers and tanks. Therefore, with urbanization more storage containers in more settlements are available favouring breeding of *An.stephensi*. Hence, due to urbanization in both the study areas percentage of *An.culicifacies* was much lower (25% to 35%) compared to *An.stephensi*. *An.stephensi* has higher percentage in urban and peri-urban of Ghaziabad and Delhi regions. *An.culicifacies* due to decreasing rainfall and increasing pollution has lesser breeding sites available. Similar results of higher percentage of *An.stephensi* compared to *An.culicifacies* were explained by Rani *et al*.,(2018).

Due to urbanization Delhi had higher percentage of *An.stephensi* than *An.culicifacies* in 2001. Habitats for *An.culicifacies* were only in areas where pollution was not in natural waterbodies. *An.stephensi* being a container and tank breeder was at advantage as it was not

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influenced by pollution (Batra *et al*., 2001; Surendran *et al*., 2019).Climate change in urbanizing cities has influenced malaria vectors which in turn influence malaria transmission. Correlation analysis of climate factors like humidity, rainfall and temperature with malaria vectors as well as malaria cases showed significant relationship. Minimum and Average temperature influence malaria cases positively in both Delhi and Ghaziabad. Rainfall and humidity positively impacts *An.culicifacies* (Figure 4) in Delhi and Ghaziabad. This may be due availability of more breeding sites for this vector with more rains. *An.stephensi* is influenced lesser due to rainfall in Delhi due to more container breeding while in Ghaziabad rainfall has higher influence as accumulation ofwater in discards and tanks as well as storage containers during rains provide breeding sites to them (Figure 4).

An.stephensi in Delhi and *An.culicifacies* in Ghaziabad are positively influenced by temperature (minimum and average). These vectors are responsible for malaria cases in their respective regions. Maximum temperature impacts malaria vectors negatively and humidity impacts malaria vectors positively. Thus, malaria vectors are influenced by rainfall, humidity and minimum temperature positively. Effect of humidity was found higher compared to other variables. Higher influence of humidity was also showed by Yang *et al*., (2010). Maximum temperature influence malaria vectors negatively in our study. However, it is reported that increase in both maximum and minimum temperature may increase malaria cases (Zhang *et al*., 2010). Climate change and hence malaria vectors affects malaria cases.

Conclusion

Urbanization in Current study has influenced malaria vectors by increasing *An.stephensi* and decreasing *An.culicifacies* which is responsible for malaria in Ghaziabad and Delhi region. Thus, it is important to study how urbanization is influencing malaria vectors which may affect competence and hence pathogen transmission. Urban people are at more risk as they are less immune to vector borne disease like malaria. Regular monitoring of malaria and its vectors is essential to prevent epidemic making urbanizing regions malaria free.

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Article 21

Synthesis, optical and Bacterial Activity of ZnO nanoparticles

Sai Chandra J, Albert Houcine Touati, Sowmya P, Murthy KVLN, Adilakshmi G and Jhansi Rani A.

Abstract

Zinc oxide nanoparticles are manufactured using a simple wet chemical precipitation approach in thispaper. The initial components are zinc nitrate and sodium hydroxide. At a temperature of around 800 degreesCelsius, zinc oxide nanoparticles are created. Two procedures are required to make nickel doped zinc oxidenanoparticles. Precipitate is generated in the first step by reducing a mixture of zinc nitrate, ferric nitrate, andstarch with sodium hydroxide solution, and the given precipitate is thermally destroyed in the second step athigh temperatures of the order of 400^0C. X-ray diffraction spectroscopy is then used to confirm thecrystallinity of the synthesised nanoparticles (XRD). Energy Dispersive X-Ray Spectroscopy is usedto determine the powder's elemental makeup (EDAX). Scanning Electron Microscopy is used toanalyze the powder's morphology (SEM). Squid Magnetometer is used to characterize the magneticproperties of nickel doped zinc oxide nanoparticles. The ferromagnetic property of the sample wasshownbythe magnetizationbehaviouratroomtemperature.

Keywords Zinc oxide nanoparticles, Nickel doped ZnO, Antibacterial activity, Squid magnetometer, SEM

1. Introduction

Zinc oxide (ZnO) is a one-of-a-kind material with a direct band gap of 3.37 eV and a high excitonbinding energy of 60 MeV. Zinc oxide has been widely used in a variety of technological applications,including thin film transistors [K.Nomura 2003], gas sensors [S.Roy,S.Basu, 2002], transparent conductor [Takashi ogi, 2009], biomedical [Zhang Y, 2013], and piezoelectricuse[Min- Hua Zhao, 2004],due toitsremarkableopticalandelectricalqualities.ForthesynthesisofZnO nanostructuredmaterials, researchers have used a variety of methods, including nonionic polymer assisted thermolysis [JoshuaW.Kriesel, 2001],conventional solid state reaction [Zhi-Peng Sun Lang Liu, 2006], sol-gel method [LubomirSpnahel, 1991], electron beam deposition [Shunichi hayamizu, 1996], an electrochemical route[ShailajaMahamuni,1999],chemicalco-precipitationmethod[MayekarJyoti, 2013], andsoon.

Chemical co-precipitation is the best of these procedures since it is easy, inexpensive, and produces ahigh yield rate. Due to their considerable potential, II–VI semiconductor materials at the nanoscale scale play acrucial role in a variety of applications.

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When doped with transition metals (TM), ZnO is an important II–VIsemiconductor exhibiting room-temperature (RT) ferromagnetism (FM) [M. Venkatesan, 2004;, K.Sato, 2001; KazunoriSato, (2000); S.Risbud, 2003]. As a result, ZnO is oneof the most promising materials for spintronics and diluted magnetic semiconductor (DMS) applications. Thegoal of this research is to use doping to improve the magnetic characteristics of ZnO nanoparticles. Theprimary difficulty for these materials is to achieve magnetic characterisation at room temperature so that theymay be used in technological applications. The chemical co-precipitation approach is used to make roomtemperature ferromagnetic micro particles in this work. The magnetic, structural, and morphological properties of the synthesized samples are studied.

2. Experimental

Zinc oxide nanoparticles are synthesized by precipitation method [MayekarJyoti, 2013]. For the synthesis of nickeldoped zinc oxide nanoparticles, 14.87gm of zinc nitrate and 0.1gm of starch is dissolved in 100 ml of distilled water. This solution is heated till the temperature reaches 60^0C. Add 1 M of sodium hydroxide solution to above solution drop by drop with continuous stirring by magnetic stirring. Prepare another solution of 2.908 gm of nickel nitrate and 0.1gm of starch in 100ml of distilled water. Add this solution to above solution and stir for two hours using magnetic stirrer. Keep the solution overnight. Precipitate is formed.Ovendry the precipitate at 100^0C. Powder is formed. Keep this powder in Muffel furnace for 6hrs at 400^0C. Nickel doped zinc oxide nanoparticles are formed. Keep this powder in Muffel furnace for 6hrs at400^0C.Nickel doped zincoxidenano particles are formed.

3. Results and Discussions

3.1. Xraydiffractionspectroscopy

The Xray diffraction (XRD) patterns of the powdered samples were recorded usingan Xpert PRO diffractometer with Cu$K\alpha$ radiation at room temperature. The crystallite size was estimated using the Scherrer equation from the full width at half maximum of the major XRDpeak.

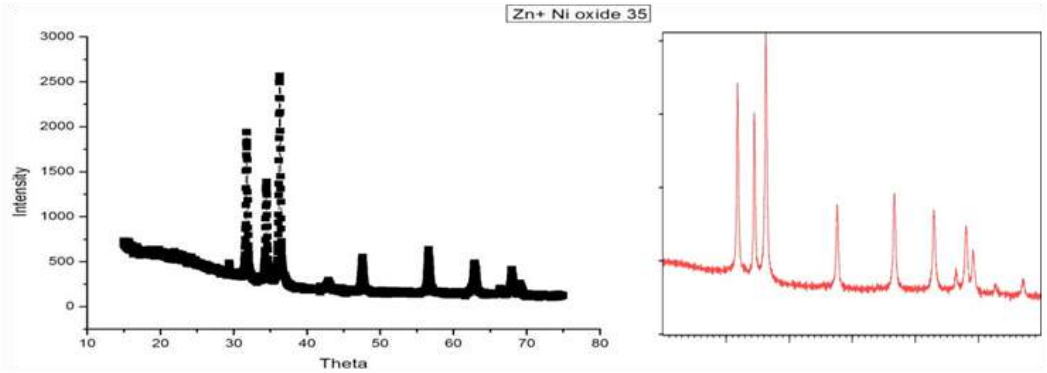

Fig1 and 2 Xray diffraction patterns of Zinc oxide and nickel doped zincoxide nanoparticles

X-ray diffraction (XRD) was used to examine the crystalline structure using a CuK radiation(1.5405) diffractometer running at 40 kV, 30 mA for angles between 2=100 and 80°

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in 0.02° in crements. The samples arevery crystalline, as seen by the crisp and strong peaks. The creation of phase pure wurtzite structure of ZnO isshown by the XRD peaks for (100), (002), (101), (102), (110), (103), and (112) planes. Table 1 shows the latticeconstants 'a' and 'c' determined from XRD data. It agrees well with the reference value (a=b=3.249, c=5.206)[JCPDS-36-1451]. There were no more peaks that corresponded to nickel oxide's subsequent phases. We maydeduce that the Ni substitution has no effect on the wurtzite structure of ZnO and that Ni2+ occupies the Zn2+site in the crystal lattice. Because of the difference in the ionic radius of the elements [r(Zn2+) = 0.60 andr(Ni2+)=0.55],the lattice constants of Zn1-xNixO(x=0.055) are some what less than those of pure ZnO (x=0.055).

3.2. Energy Dispersive X ray Spectroscopy

Figures 3 and 4 show the energy dispersive X-ray analysis of pure zinc oxide nanoparticles and nickel doped zincoxide nanoparticles.TheX-ray patterns show thatallof the dopants are present in the respective spectrum. Further more, it is significant to note that no alien components are present in the spectrum. It's anextra layer of assurance for the samples' purity. Tables 2 and 3 provide the quantitative analysis of all the samples. Inaddition, there are no impurities discovered in the table.

Lattice constant	Pure ZincOxide	Nickeldoped zincoxide
a(A^0)	3.250	3.248
c(A^0)	5.209	5.208

Table 1 Lattice parameters of Pure ZnO and Nickel doped ZnO nanoparticles

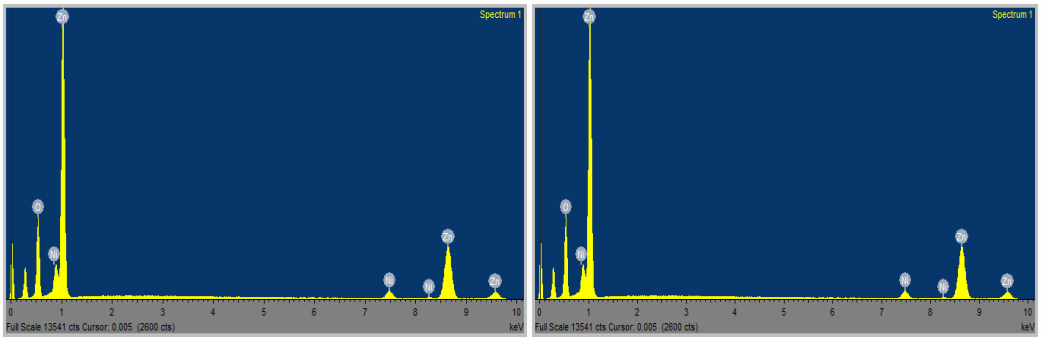

Fig3 and 4 EDAX spectrums of Pure and Nickel doped ZnO nanoparticles

Spectrum	Zn	O	Ni
ZnO	67.96	31.84	------
Nickeldoped ZnO	36.57	60.88	2.56

Table 2 Elemental Composition of doped and undoped Zincoxide nanoparticles

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3.3. Scanning Electron Microscopy

The zinc oxide nanoparticles feature flower-like forms in SEM pictures, but the nickel doped zincoxide nanoparticles are spherical in nature. The zinc oxide flower-like structures have a diameter of 20 nmand a length of 200 nm, whereas the produced nickel doped ZnO NPs have a diameter of 50 nm. These dimensions correspond to tho sedetermined using the Debye Scherrer formula.

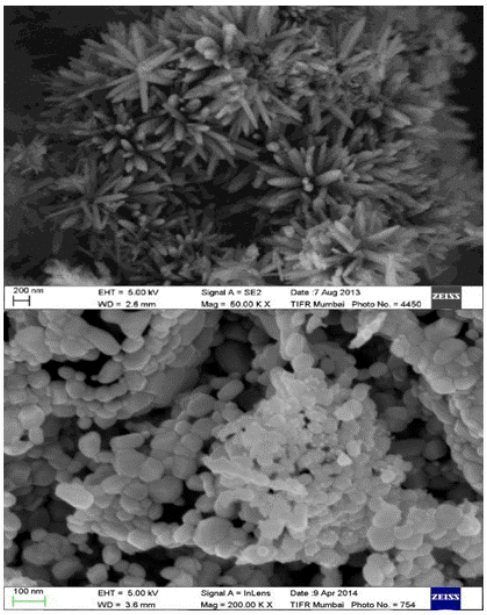

Fig5 and 6 SEM images of Pure ZnO and Nickel doped ZnO NPs

3.4. Ferromagnetism In Nickel Doped ZincOxide Nanoparticles

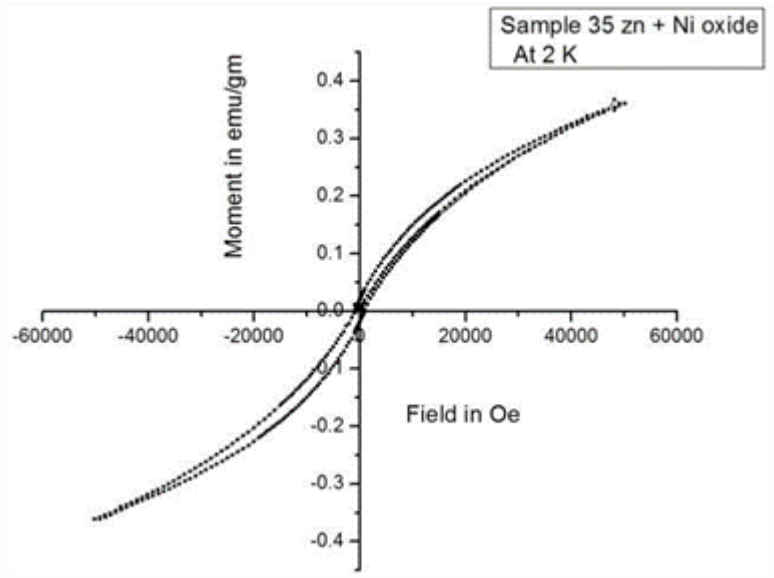

Fig7 BH loop of nickel doped ZnO Nps

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Squid magnetometer is used for magnetic characterization. The sample exhibits ferromagnetic behaviour, according to the BH loop research.The saturation magnetization is 0.37emu/gm, according to the results.

Conclusions

Due to the presence of defect-related mechanisms such as oxygen vacancies, ferromagnetism is found.Nickel doping causes the Zinc oxide nanoparticles to become ferromagnetic. The present work's Ni doped ZnO nanoparticles, which have a low magnetism, could be used to make diluted magnetic semiconductors for spintronic applications.

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Article 22

A Overview of Caprine Coccidiosis in Goat

Tejswini A. Sontakke and Dinesh Nalage

Abstract

The illness coccidiosis, caused by a coccidian parasite of the genus Eimeria, causes significant economic loss in tiny ruminants. Out of 16 Eimeria species, including *E. arloingi*, *E. ninakohlyakimovae* and *E. christenseni*, in the juvenile and adult goat groups, are the most pathogenic. Weaning, bad weather, dietary changes travel, and regrouping are all important factors in small ruminant coccidiosis.Several species of the genus Eimeria cause this protozoan infection, which develops in the small and large intestine.Because the incidence and quantity of caprine Eimeria spp. is so high, efficient infection management is critical to avoid clinical coccidiosis in goatherds.

Keywords Eimeria, Coccidiosis, Goat, Infection

Introduction

, immunity, diagnosis and control of intestinal coccidiosis in goatsMore than one million goats are now raised globally, with a significant rise in headcount projected in the coming years due to the increasing market for goat milk and meat.With an overallpopulation148.9 million head of goats and 74.3 sheep , India is 3rd largest country in the world. Due to their capacity to graze near to the ground, pinch food, and ingest ligneous products, goats may flourish in unfavorable situations such as arid places with little feed.Coccidiosis in sheep and goats is commercially important infections impacting the sector globally.Caprine coccidiosis caused by the apicomplexan protozoan Eimeria spp., which is widespread and common in goat herds. It is most common and economically significant illnesses in goat herds. (Agyei et al., 2004; Hashemnia et al., 2014; Norton, 1986; O'Callaghan, 1989). Coccidiosis severity is determined by Eimeria species and the amount of the infectious dosage of oocysts. (Pattison, M., B. Mcmullin and Alexander, 2009). Out of the 16 species of Eimeria of goat *E. arloingi*, *E.christenseni* and *E. ninakohlykimovae* are highly pathogenic Eimeria species of goat.(Sontakke 2016). Coccidiosis is now controlled by better management methods in conjunction with anticoccidial medications used as a metaphylactic therapy. (Ruiz et al., 2012). This chapter reviews the prevalence, pathology, pathogenesis

Live Stock Population in India by Species (Millions Numbers)

Species	2003	2007	2012	2019
Cattle	185.2	199.1	190.9	192.5
Adult Female Cattle	64.5	73.0	76.7	81.4

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Buffelo	97.9	105.3	108.7	109.9
Adult Female Buffelo	51.0	54.5	56.6	55.0
Sheep	61.5	71.6	65.1	74.3
Goat	124.4	140.5	135.2	148.9
Poultry	489.0	648.8	729.2	851.8

*** Includes chicken, ducks, turkey & other birds**

(https://www.nddb.coop/information/stats/pop)

Coccidiosis

Main illness in sheep and goats is coccidiosis, caused bygenus Eimeria(protozoans) that grow in the small and large intestines. They are an intracellular parasite in the epithelium of the gastrointestinal system, and the sickness is self-limiting. They are very species specific, which means that Eimeria species infect sheep will not infect goats or cattle, and vice versa.

Economic importance

Coccidiosis is economically significant losses caused by clinical illness (diarrhoea) and subclinical infections (poor weight gain in particular).Coccidiosis can become subclinical infection of great economic importance(Sontakke 2015).These losses can be attributed to decreased output in the event of mild infection without clinical indications, or to the direct effects of diarrhoea on animal development and death in the event of clinical coccidiosis. (Foreyt, W.J 990, Chartier 2012). Coccidiosis is regarded as one of the most commercially significant illnesses in the world's intensive sheep and goat industries. (Varghese and Yayabu, 1985; Chhabra and Pandey, 1991).

Historical Background

The first Apicomplexan protozoan was seen by Antony Van Leeuwenhoek (1674) who saw oocysts of *Eimeria stiedae* in the gall bladder of rabbit. However after 150 years Hake (1839) first described as globules of pus associated with an infected liver. Luckart (1879) first recognised the organism as independent forms and gave the generic name coccidian. Most of the coccidian parasite can only be seen under the microscope. Luhe (1902) suggested the name coccidia as well as *Eimeria* are the same and that *Eimeria* should have priority. In recent times the *Eimeria* has been accepted as the generic name for these organisms. The parasites have life cycle involving three phases: schizogony (also known as merogony), gametogony and sporogony (or sporulation). Twentieth century,its classification in the was complicated by Apicomplexa's widespread spread and the diversity of its species.(Lefevre, B., 2010)

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Taxonomical Classification

The word Protozoa was first used to describe a taxonomic class in 1818, although successive categorization methods raised the group to higher levels, including phylum, subkingdom, and kingdom. Protozoa is classified as a kingdom in numerous categorization schemes suggested by Thomas Cavalier-Smith and his coworkers since 1981.These are mainly microscopicunicellular organismsin which the various activities of metabolism, locomotion are carried out by organelles of the cell.

Classification of *Eimeria*:

Domain – Eukaryota

Infra- Kingdom – Animalia

Kingdom – Protista (Protozoa) (Goldfuss, 1818)

Sub-kingdom – Biciliata (Cavalier Smith, 2004)

Infra- kingdom – Alveolata (Cavalier Smith, 1991)

Phylum – Myzozoa (Cavalier Smith and Chao, 2004)

Sub-Phylum – Apicomplexa (Cavalier Smith, 2004)

Class – Conoidasida (Levine, 1988)

Sub- class – Coccidia or Coccidiasina (Leayckart, 1879)

Order – Eucoccidiorida (Ledger and Dubosq, 1910)

Sub -order – Eimeriina (Ledger, 1911)

Family – Eimeriidae (Minchin, 1903)

Genus – *Eimeria* (Scheinder, 1875)

Fig. 1: Taxonomic classification of coccidia [Soulsby, 1982, Sontakke 2016]

Sr. No	Coccidian name	Infection Site	Prepatent period (days)	Pathogenicity
1	*E.arloingi*	Small intestine	20 days	++ high
2	*E.christenseni*	Small	14-23	++ high

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		intestine		
3	*E.ninakohlyakimovae*	Small and Large intestine	10-13	++ moderate
4	*E.caprina*	Small and Large intestine	17-20	++ moderate
5	*E.Parva*	Small and Large intestine	12-14	++ low
6	*E. aspheronica*	Unknown	14-17	low
7	*E. hirci*	Unknown	13-16	moderate
8	*E. jolchijevi*	Unknown	14-17	low
9	*E. caprovina*	Unknown	14-20	low
10	*E. alijevi*	Small intestine	7-12	low
11	*E. ahsata*	Small intestine	18-20	-
12	*E. faurei*	Small and Large intestine	9-12	-
13	*E. intricata*	Small intestine	-	-
14	*E. straightatus*	Unknown	Unknown	Unknown
15	*E. parbhaniensis*	Unknown	Unknown	Unknown
16	*E. susheelensis*	Unknown	Unknown	Unknown

Table 1: Pathogenic species of Eimeria in goat ((Taylor et al., 2007)

Etiology

Species of *Eimeria*

E. christenseni, E. arloingi, E.caprina, and *E. ninakohlyakimovae* are reported as the highly pathogenic speciesspecies in goat.The 16 species of Eimeria have been described from goats in India, *E. ahsata, E. arloingi, E. crandallis, E. parva, E. ninakohlyakimovae, E. christenseni, E. intricata, E. parbhaniensis, E. hirci, E. straightatus, E. jolchijevi* and *E. susheelensis, E. aspheronica, E. caprina, E. caprovina, E. faurei* , Out of four species *i.e. E. arloingi, E. crandallis, E.ninakohlyakimovae,* and *E. parva* have been recorded by all Indian workers in different region. (Nikam 1983, More 2011,Sontakke 2016)

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Species %	**Sample No.**	*E. arloingi,*	*E. crandallis*	*E. ninakohlyakimovae*	*E. parva*
Nikam 1983	575	12.52	3.83	2.44	2.78
Jadhav 2002	297	5.72	2.69	2.02	4.72
More 2011	2636	3.03	2.73	2.23	2.4
Sontakke 2016	3004	24.67	28.63	29.09	29.86
Singh 2020		42.0	-	26.5	-

Table 2: Species prevalence in Goat

Morphological Characteristics of Eimeria Species in Goat

It has been reported by many countries around the world. Sixteen Eimeria spp. were describedas etiological agents in goat. Identification of the species based on their morphological characteristics of oocysts (color, form index, shape, absence or presence of residual, absence or presence of micropyle and its cap, Stieda bodies and its polar nature) and time of sporulation Coudert's key (Coudert, 1992, Nikam 1983). Table no.3.

Species	**Ooocyst (µm)**			**Sporocyst (µm)**			**Polar granules**	**Sporocystic residium**	**Shape of sporozoite**	**Steida body**	**Refractile globule**
	shape	**Length**	**Width**	**shape**	**Length**	**Width**					
E. ahsata	Elongated , ellipsoid	22.0-40.0	18.0-28.0	Elongated, ovoid	10.0 -16.0	4.0 -10.0	present or absent	Form of several granules	Elongate	Absent	1 small, 1 large
E. intricata	Ellipsoid, elongate ovoid	35.0-53.0	24.0-40.0	Elongate, ovoid	10.0-20.0	8.0-16.0	Absent	Granular and scattered	Fusiform	Button like	1 large , 1 small
E.faurei	Ovoid/Egg shaped	27.0-41.0	21.0-35.0	Broadly ovoid, pyriform	10.0-16.0	7.0-10.0	Absent	Form of granules.	Elongated	Clearly seen	2 clear refractile globule
E. ninakohlyakimovae	Subspherical	20.0-34.0	16.0-28.0	Ovoidal, spindal	9.0-18.0	5.0-10.0	Present	Many scattered granules	Elongate	Small steida body	1 small, 1 large
E. parva	Subspherical	16.0-29.0	15.0-29.0	Elongate	15.0-9.0	8.0-5.0	Absent	Present	Ovoid	Absent, some times present	1 large, 1 small
E. arloingi	Ellipsoid, elongate	29.0-45.0	18.0-34.0	Elongate, Ovoid	11.0-15.0	7.0-11.0	Absent	Granule form	Elongate	Present	1 small 1 large
E. crandallis	Ellipsoidal, ovoidal	20.0-33.0	18.0-26.0	Elongate , ovoid	10.0-15.0	4.0-8.0	Present	Granular residuum	Elongate	Absent	2 refractile globules.
E. christenseni	Ovoidal, ellipsoidal	30.0-44.0	20.0-31.0	Broadly ellipsoid	14.0-24.0	10.0-17.0	Absent	form of granules	Elongate	Absent	1 small 1 large
E. jolchijevi	Ellipsoid, ovoid	28.0-38.0	20.0-27.0	Elongate, ovoid	10.0-17.0	6.0-10.0	Present	Oval granular form	Elongate	Present	2 large
E. parbhaniensis	Egg shaped, elongate ovoid	29.0-52.0	22.0-39.0	Elongate ovoid	10.0 –18.0	7.0-12.0	Present	form of spherical granules	Elongate	Button like	1 small, 1 large
E. caprina	Ellipsoid, slightly ovoid	27.0-38.0	18.0-25.0	Elongate, ovoid	10.0-15.0	8.0-12.0	one or more	Present	Elongate	Prominent Steida body	1 small, 1 large
E. caprovina	Ellipsoid, subspherical	25.0-32.0	21.0-25.0	Elongate, ovoid	11.0-16.0	7.0-10.0	one or more	form of granules	Elongate	Small stieda body	2 large clear globules
E. striatatus	Oval	24.0-40.0	20.0-36.0	sporocysts spindle shaped	12.0-18.0	7.0-11.0	Absent	Spherical large granules	Kidney shaped	Present	1 small 1 large
E. hirci	ellipsoid, ovoid	17.0-28.0	13.0-23.0	Elongate, ovoid	9.0-14.0	6.0-9.0	1 or more	few scattered granules	Elongate	Present	1 small, 1 large
E. aspheronica	broadly ovoid, oval	25.0-37.0	19.0-27.0	Pyiriform, ellipsoid	10.0-15.0	7.0-10.0	Prominent	scattered granules	Elongate	crescent shaped	2 large clear globuules
E. Susheelensis	Oval to egg shaped	16.0-22.0	14.0-18.0	Broad, elongate	9.0-14.0	5.0-10.0	Absent	Present	Bean shaped	Conspicuous stieda body present	refactile globuules

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Table 3: Morphological characterstics of *Eimeria* species (Sontakke 2016)

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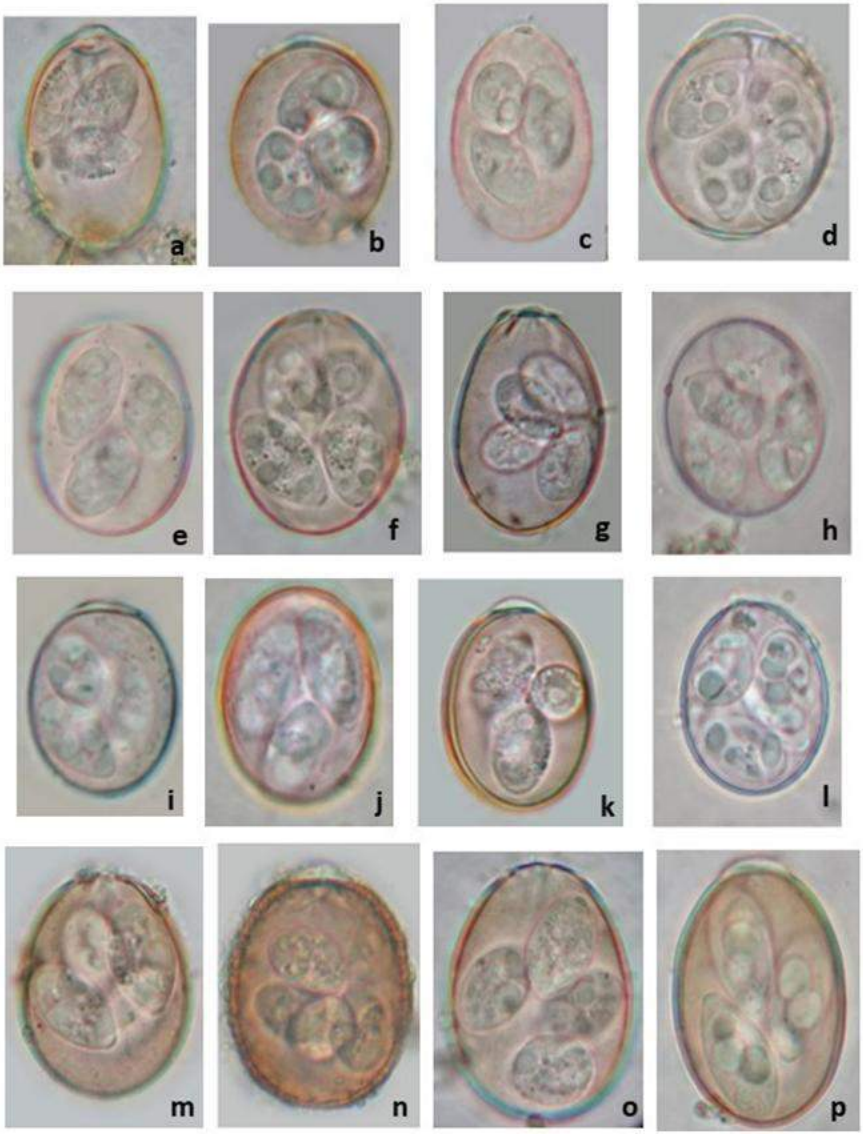

Fig. 2 Oocysts of *Eimeria* species of goats as identified microscopically (1000X) after sporulation, A. *Eimeria ahsata*, B. *Eimeria crandallis*, C. *Eimeria faurei*, D. *Eimeria jolchijevi*, E.*Eimeria caprina*, F. *Eimeria caprovina*, G. *Eimeria christenseni*, H. *Eimeria parva*, I. *Eimeria hirci*, J.*Eimeria ninakohlyakimovae*, K.*Eimeria arloingi*, L. *Eimeria susheelensis*, M.*Eimeria apsheronica*, N. *Eimeria intricata*, O. *Eimeria straightatus*, P. *Eimeria parbhaniensis.* (sontakke 2016)

Life Cycle:

The life cycle of Eimeria species is homoxenous requiring only one host. It includes an exogenous phase of maturation of the oocyst (sporogony), which occurs outside the

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host, and a parasitic endogenous phase within the host with an asexual followed by a sexual multiplication (Soulsby, 1982). Sporulated oocysts are formed after 2–7 days according to the species of Eimeria and the environmental conditions; moisture, oxygen and temperature are particularly important.

Unsporulated oocysts are passed in faeces from infected goats. When goats consume sporulated oocysts, the schizogony phase begins.Bile and trypsin activity causes the release of eight sporozoites from each oocyst. Sporozoites enter intestinal cells and divide multiple times to become trophozoites, which then divide again to become schizonts (meronts).When the schizont ruptures, the merozoites contained within is released into the intestinal lumen; each of the merozoites penetrating a new intestinal cell. It is then followed by another asexual cycle or sexual cycle, depending on the species involved.

One of the three cycles may occur prior to gametogony. Microgametocytes (male) and macrogametocytes (female) grow into microgametes and macrogametes, respectively, during gametogony. The microgametes are discharged into the intestinal lumen, where they fertilise intracellular macrogametes, resulting in zygotes or oocysts. Host cells break, releasing unsporulated oocysts into the intestinal lumen, and oocysts depart the host through faces. The full life cycle takes two to three weeks in the host and can last several months.(fig. 3)

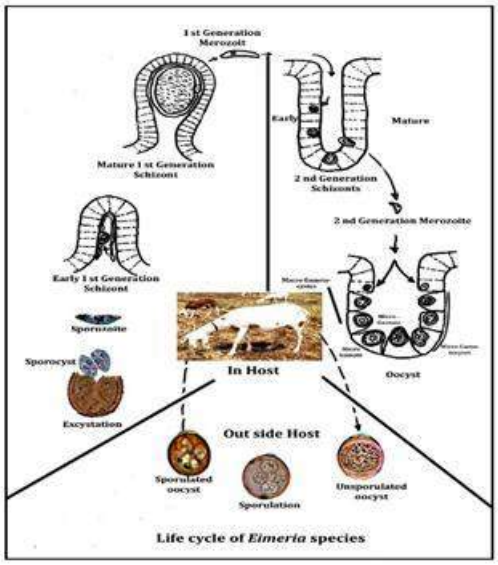

Fig 3: Life Cycle of *Eimeria*

Epidemiology :

Coccidiosis is a disease that affects tiny ruminants and is found all over the world. Coccidiosis is a serious subclinical illness that can result in large financial losses in the small ruminant business. Adult animals are disease resistant, but not completely immune to

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infection. Disease development is influenced by a variety of variables, including husbandry and management (Taylor 2007) (Fig.4).

Since the amount of sporulated oocysts represents the infective stage of the parasite, sporulation of oocysts expelled by faeces in the environment is vital in contemporary production, children are born into potentially extremely contaminated environments. In most parts of the world, there is evidence that some oocysts survive the winter. (1986, Foryet). Coccidiosis is a serious subclinical illness that can result in large financial losses in the small ruminant business (Kusiluka 1996).

Temperature, moisture, and oxygen tension are the primary determinants of coccidial oocyst survival and progression to the infective stage. The ideal temperature for most Eimeria spp. oocysts in goats to sporulate is 28-31°C. In the external environment, sporulated oocysts demonstrate a high level of resilience (they can survive several months or even more than a year). Extreme desiccation and direct sun exposure, on the other hand, reduce oocyst life, and temperatures below 30°C or over 63°C are deadly. (Foreyt, 1990)

The humid tropics' climatic conditions are ideal for coccidian survival and growth throughout the year(Kusiluka 1996).

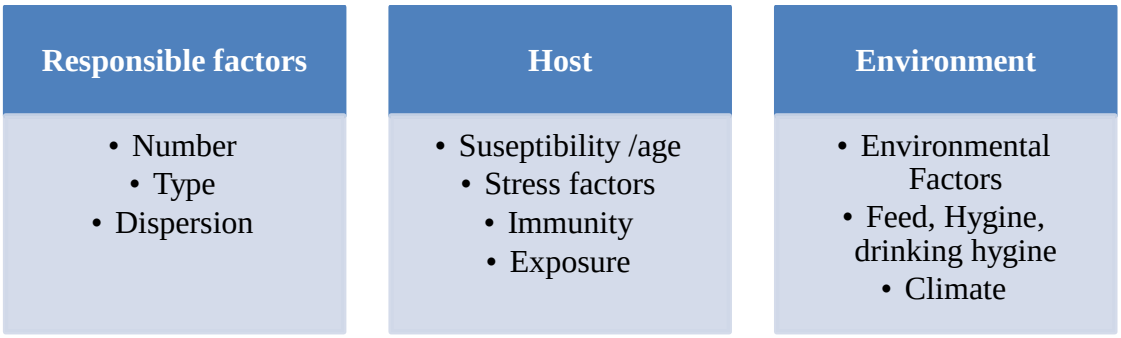

Fig. 4: Factors responsible to occurrence of Coccidiosis [(BAHC), 2008]

Pathogenesity

The most prevalent species were *Eimeria arloingi*, *E. ninakohlyakimovae* and *E. hirci* in goat. The *E. ninakohlyakimovae* has been recognized to be more pathogenic in goat population (Chartier and Paraud, 2012), *Eimeria arloingi* causes focal mucosal hyperplasia and polyp formation, *Eimeria caprina* causes mucosal destruction in both (small and large) intestines &*Eimeria hirci*and *Eimeria christenseni*are also possibly pathogenic (Andrews, 2013). *E. ninakohlyakimovae*, *Eimeriaarloingi* and *E.christenseni* produced abdominal pain, inappetence, bloody diarrhea and papilloma like lesions in the intestine of young kids (Yvore et al., 1980). As per most of the earlier reports, healthy goats may resist Eimeria infections duly well without establishment of clinical signs but stressors might be breaking the host

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parasite relationship leading to economic losses. Although, development of a positive relationship between parasites which is in tandem with the role of contamination in the acquirement as seen with coccidial as well as helminthes parasites (Kanyari, 1993)

Eimeria species that infect and kill the crypt cells of the intestinal mucosa are the most harmful.Because of that most of the animal's small intestine severe damage by the coccidial infection.

Its pathogenicity depends on the various site of infection in the host. Because of the coccidial infection changes occur in intestinal mucosa that effects increased the rate malabsorption, diarrhea. Diarrhea leads to dyhaydration, acidosis, anaemia, abdominal pain, anorexia and terminal shock.

Clinical signs

When optimal conditions for the development of pathogenic species occur, common clinical signs of coccidiosis are usually observed: anemia, weakness, depression, abdominal pain, lethargy, anorexia, dehydration, coarse hair, poor weight gain, low conversion of food and pasty stools without blood streaks. In addition, in animals heavily infected with species that develop gamonts in the large intestine such as E. ninakohlyakimovae, accompanying all clinical signs previously mentioned, a severe liquid diarrhea can be observed, sometimes hemorrhagic, capable of dragging portions of intestinal mucosa. (Dai et al. 2006 , Ruiz et al. 2013, Cox ,1998)

Diagnosis

Although, traditional morphological identification is still useful. The identification was performed by the comparison with original parasites and somewhat variations in size of oocysts (length and width) might be found, and this variation was considered as a common issue by previous studies (Hassum and Menezes, 2005; Bhatia et al., 2010). Most of the caprine Eimeria spp. Can be differentiated by an experienced examiner by the morphology of the unsporulated and sporulated oocysts. Study of sporulatedoocyst is more desirable than unsporulatedoocyst.Most probably, different Eimeria spp. are present in all animals of the herd, but not all of them may be pathogenic species, so the simple presence of oocysts in feces is not a sufficient reason for the diagnosis of goat coccidiosis (Koudela and Bokova 1998).

Treatment

Anticoccidian medicines may be of limited benefit in individual active instances. The majority are used as coccidiostats, which prevent but do not eliminate coccidiosis. As a result, the primary purpose of a coccidiostat medicine is to limit the number of new cases in a group of animals. (Vihan, 2010). Drugs used for treatment of coccidiosis include sulfonamides, nitrofurans, gonophores' antibiotics and quinolons. Nitrofurans, Amprolium,

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Salinomycin, Toltrazuril, clopidol and methy benzoquate use for the control of infection.Sulfonamides, Nitrofurazone these drugs control of secondary bacterial infection.

Prevention

Animals confined indoors on moist bedding polluted by intensively stocked pastures are especially vulnerable to coccidiosis, especially in cold, wet weather. Disease incidence can be minimised by avoiding overcrowding and stress, as well as practising good cleanliness. Raising feed and water troughs can help to avoid contamination by lowering infection levels.

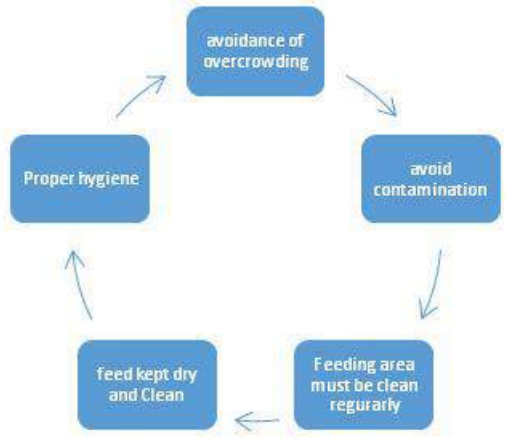

Fig. Prevention factors for avoiding coccidial infection

Conclusion

E. ninakohlyakimovae, E. arloingi and *E. christenseni* produced abdominal pain, inappetance, bloody diarrhoea and papilloma like lesions in the intestine of young kids (Yvore et al., 1980). Different species of *Eimeria* demonstrate different tissue and organ specificity in the infected host. Coccidiosis is an intracellular disease, which is caused by *Eimeria* species that cause morbidity and mortality in goat. The occurrence of coccidiosis is due to highly contaminated environment, Because of that regular examination is very important. *Eimeria* species impact on the intestinal tract and damage the epithelial cells of the intestine.

There should be proper management of sheep and goats to avoid pre disposing factors for the occurrence of the disease. Try to reduce the disease incidence through avoidance of overcrowding and stress. Attention should be given for the hygienic system of housing and feeding. Prevention should be given more attention than treatment.

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